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Restriction Analysis of β-Tubulin Gene for Differentiation of the Common Pathogenic Dermatophytes

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Background: Identification of dermatophytes at the species level, relying on macro- and microscopic properties of the colonies is time-consuming, questioned in many circumstances, and requires considerable expertise. In this study, we examined the potency of a new genetic marker, β-tubulin (BT2) gene, for differentiation of dermatophytes in an in silico and experimental restriction fragment length polymorphism (RFLP) profile. *Methods:* The BT2 sequences of dermatophyte species were retrieved from GenBank and analyzed using bioinformatics softwares to choose suitable restriction enzyme(s). Forty reference culture collections and 100 clinical isolates were PCR-amplified using the primers T1 and Bt2b and consequently subjected to virtual RFLP analysis. The dermatophytes were identified according to specific lengths of bands in agarose gel electrophoresis. *Results:* After digestion of partially amplified β-tubulin gene with the restriction enzyme *Fat*I, three dermatophyte species, that is, *Microsporum gypseum*, *M. canis, and Trichophyton verrucosum* yielded unique restriction maps while the remaining species including *T. interdigitale, T. rubrum, T. tonsurans, T. schoenleinii,* and *T. violaceum,* were identified by further restriction digestion by *Alw*21I, *Mwo*I, and *Hpy*CH4V endonucleases. The length of RFLP products was same as of those expected by computer analysis. *Conclusion:* The two-step BT2 restriction mapping used in this study is an effective tool for reliable differentiation of the clinically relevant species of dermatophytes. J. Clin. Lab. Anal. 28:91–96, 2014.

Key words: dermatophytes; β-tubulin gene; identification; RFLP

INTRODUCTION

Dermatophytes comprise a group of closely related fungi, correlating to three anamorphic genera of *Trichophyton, Microsporum,* and *Epidermophyton*, each includes several distinct species. They can colonize or infect the keratinized tissues (skin, hair, and nails) of humans or animals, causing clinically localized to extensively generalized lesions termed as dermatophytosis (1, 2). From etiological, therapeutic, and epidemiological standpoints, identification of the clinically isolated dermatophytes at the species level is important to verify the diagnosis, organize the appropriate therapy, establish the preventive strategies for infection, as well as to extend our knowledge in the realm of dermatophytes ecology and epidemiology (2, 3). The current scenario for species identification of these fungi relies on macromorphology and microscopic properties of the colonies on specific media like Sabouraud's glucose agar with cycloheximide and chloramphenicol, along with analysis of some nutritional requirements or biochemical characters (1, 3). However,

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due to the high similarity and variability in morphology, the pleomorphism phenomenon, and a long time required for emergence of phenotypic characteristics, identification of these molds based on phenotypic criteria is time-consuming, requires a significant degree of experience, and remains imprecise in many circumstances (4–6). During the last decades, the development of molecular biology techniques has provided more rapid and precise alternatives for species delineation of dermatophytes and in this context, molecular targets like internal transcribed spacer (ITS) regions of rDNA (3, 7, 8), DNA topoisomerase II (9), chitin synthase 1 (CHS1; 10, 11), and actin genes (12) have met with some success. In the present study, we conducted a PCR-RFLP (PCR-restriction fragment length polymorphism) assay on β-tubulin (BT2) gene aiming to evaluate the efficacy of this new marker for accurate differentiation of pathogenic dermatophytes, along with special impression on the species of clinical relevance in Iran.

MATERIALS AND METHODS

Computer-Simulated RFLP Analysis

The BT2 sequences for dermatophyte species were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/pubmed/), exported to the MEGA 4 software (http://www.megasoftware.net), and aligned with T1/Bt2b primers. Each related trimmed sequence was exported to DNASIS program (Hitachi DNASIS® MAX version 3.0) for in silico digestion with all restriction endonucleases included in the software. The enzymes presenting the best-resolved species-specific RFLP patterns were chosen as candidates for next evaluations.

Fungal Strains and Growth Conditions

The PCR-RFLP was optimized using 40 culture collections and 100 clinical isolates. The reference strains were kindly prepared by Teikyo University Institute of Medical Mycology (TIMM) and the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). The reference strains were *T. interdigitale* (CBS 130788, CBS 130789, CBS 130790, CBS 130791, CBS 130792, CBS 130794, CBS 130796, CBS 130799, CBS 130801, CBS 130803, CBS 130804, NBRC 5809, NBRC 5466, NBRC 5812, NBRC 5974), *T. schoenleinii* (NBRC 8192, NBRC 8191), *T. tonsurans* (CBS 130800, CBS 130814, NBRC 5928, NBRC 5945), *T. rubrum* (CBS 130927, NBRC 5808, NBRC 5467), *T. violaceum* (CBS 130937, NBRC 31064, CBS 376.92), *T. verrucosum* (CBS 562.50, CBS134.66), *E. floccosum* (CBS 130793, CBS 130802, NBRC 9095, NBRC 9045), *M. gypseum* (CBS 130939, IFO 5948, IFO 8228), and *M. canis* (CBS 130795, CBS 130797, CBS 130798, NBRC 9182). All strains were cultured on Mycobiotic Agar (Difco, Detroit, MI, USA) and incubated at room temperature for 3 weeks. The identity of all used clinical isolates, including *T. interdigitale* ($n = 37$), *T. rubrum* ($n = 25$), *T. schoenleinii* ($n = 2$), *T. violaceum* ($n = 1$), *E. floccosum* ($n = 10$), *T. tonsurans* ($n = 10$), *M. canis* ($n = 13$), and *M. gypseum* ($n = 2$) was delineated by ITS-RFLP and sequencing (6).

DNA Isolation

Total DNA of each strain was extracted from the colony using the previously described method (13). Briefly, a small plug of young colony was put into an 1.5 ml tube containing 300 μl of lysis buffer (200 mM Tris-HCl (pH 7.5), 25 mM EDTA (Ethylenediaminetetraacetic Acid), 0.5% w/v SDS (Sodium Dodecyl Sulfate), 250 mM NaCl), and crushed with a grinder and mixed with phenol-chloroform (1:1), vortexed in few seconds, and centrifuged at 10,000 rpm for 10 min. Then the supernatant was mixed with chloroform and centrifuged. The DNA was precipitated with 1/10 volume of 3.0 M sodium acetate and equal volume of iso-propanol at −20°C for 10 min, washed with 70% ethanol, dried, and suspended in 50 μl of ultrapure water.

PCR Reaction

The universal primers T1 (5′-AACATGCGTGAGATT GTAAGT-3′) (14) and Bt2b (5′-ACCCTCAGTGTAGT GACCCTTGGC-3′) (15) were used for partial amplification of BT2 gene. PCR reactions consisted of 2 μl template DNA, 2.5 μl 10× reaction buffer, 1.5 mM $MgCl_2$, 400 μM deoxynucleotide triphosphates, 1.25 U *Taq* DNA polymerase, 25 pmol of each forward and reverse primers, and enough water up to a final volume of 25 μl. Thermal cycling program was an initial denaturation at 94°C, followed by 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C for 35 cycles and a terminal extension of 72°C for 10 min.

Experimental Validation of Restriction Analysis

To verify the computationally calculated restriction patterns, the BT2 PCR amplicons were preliminarily subjected to cleavage with *Fat*I (New England Biolabs Ltd., NHitchin, UK). For differentiation of *T. interdigitale*/*T. tonsurans* and *T. schoenleinii*/*E. floccosum* strains, which had the same RFLP profile with *Fat*I, two new digestions were performed by the enzymes *Alw*21I (Fermentas, Vilnius, Lithuania) and *Hpy*CH4V (New England Biolabs Ltd.), respectively. Digestion reaction was carried out in a mixture containing 1.5 μl of 10× buffer, 5 U of each enzyme, 5 μl of PCR product, and ultrapure water to a

final volume of 15 μl. Incubation temperature was based on the enzyme manufacturer's instructions.

Assessment of Length Polymorphisms by Electrophoresis

Ten microliters of amplified products or digested fractions were separated by electrophoresis through an 1.5 and 2% agarose gel (w/v), respectively, for 60 min at 100 V in TBE buffer (Tris 90 mM, boric acid 90 mM, EDTA 2 mM) containing ethidium bromide stain (0.5 μg/ml) and visualized under a UV light. A 100 base pair (bp) DNA ladder was used as molecular size marker in each run. Identification of the strains was performed by comparing the restriction maps observed in experimental RFLP with those deduced from in silico analysis.

RESULTS

Sequence analysis by MEGA 4 and DNASIS programs indicated that polymorphism in the BT2 sequences was enough to develop a PCR-RFLP profile for identification of common pathogenic dermatophytes. Additionally, except *T. interdigitale*, no intraspecies sequence variation was found among strains of each species (data not shown). The results of BT2 sequence analysis were summarized in detail in Table 1. Partial amplification of BT2 gene by the primer T1 and Bt2b yields product range from 770 bp for *M. canis* to 798 bp for *M. gypseum, T. schoenleinii,* and *T. verrucosum*. Among more than 600 restriction enzymes included in DNASIS software, four restriction enzymes, namely, *Fat*I, *Alw*21I, *Mwo*I, and *Hpy*CH4V were selected as the best enzymes for discrimination of pathogenic dermatophytes. These enzymes were used in a two-step RFLP set. In the first round, both computer-assisted and experimental digestion by *Fat*I, *M. gypseum*, *T. verrucosum,* and *M. canis,* produced specific unique restriction maps while the other species shared fragments with similar length (Table 1, Fig. 1). The remaining species were undertaken with further restriction analyses, therefore the enzymes *Alw*21I, *Mwo*I, and *Hpy*CH4V were selected as the best candidates for discrimination between *T. interdigitale*/*T. tonsurans*, *T. rubrum*/*T. violaceum,* and *T. schoenleinii*/*E. floccosum* pair species, respectively. As illustrated in Figures 2 and 3, electrophoresis of fragmented PCR products on agarose gel confirmed that BT2 digestion with *Alw*21I is clearly discriminative for *T. interdigitale* and *T. tonsurans*, while enzymatic cutting of the products with *Hpy*CH4V helps the differentiation of *T. schoenleinii* and *E. floccosum*. Hence, in the BT2-RFLP profiles by selected enzymes, 40 culture collections and 100 clinical isolates produced electrophoretic patterns congruent with expected species-specific schema achieved from sequence analysis.

TABLE 1. Results of Computer and Experimental BT2-RFLP Analysis With *Fat*I, *Alw*21I, *Hpy*CH4V, and *Mwo*I for Differentiation of Dermatophyte Strains Tested in This Study

Species	BT2 GenBank accession number used for in silico analysis	Total number of tested strains in this study	Size of the BT2 amplicon (bp)	BT2 profile after digestion with *FatI* (bp)	BT2 profile after digestion with *Alw*21I (bp)	BT2 profile after digestion with *Hpy*CH4V (bp)	Profile after digestion with *Mwo*I	Species identification by BT2-RFLP/sequencing
T. interdigitale	JF731050–JF731054	55	790	2, 202, 246, 340	152, 217, 421	NA	NA	*T. interdigitale*
T. tonsurans	JF731070–JF731074	9	790	2, 202, 246, 340	217, 573	NA	NA	*T. tonsurans*
T. rubrum	JF731063–JF731067	28	796	2, 63, 125, 246, 360	NA	NA	9, 97, 180, 510	*T. rubrum*
T. violaceum	JF731088–JF731089	5	796	2, 63, 125, 246, 360	NA	NA	9, 98, 689	*T. violaceum*
M. gypseum	JF731096–JF731098	5	798	2, 124, 672	NA	NA	NA	*M. gypseum*
M. canis	JF731101–JF731103	10	770	2, 68, 111, 117, 226, 246	NA	NA	NA	*M. canis*
T. schoenleinii	JF731081–JF731082	2	798	2, 246, 550	NA	83, 118, 125, 219, 253	NA	*T. schoenleinii*
E. floccosum	JF731122–JF731123	24	789	2, 246, 541	NA	81, 219, 489	NA	*E. floccosum*
T. verrucosum	Undeposited data	2	798	124, 250, 424	NA	NA	NA	*T. verrucosum*

NA, not applicable.

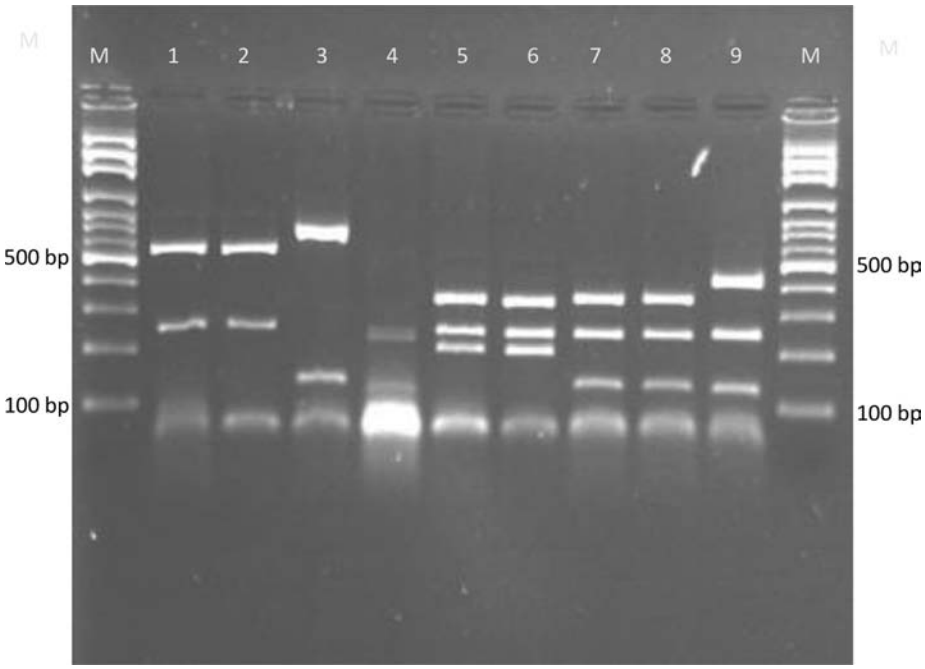

Fig. 1. Restriction profiles of BT2 gene by *Fat*I for the common pathogenic dermatophytes. Lane 1, *T. schoenleinii* (NBRC 8192); lane 2, *E. floccosum* (NBRC 9045); lane 3, *M. gypseum* (NBRC 8228); lane 4, *M. canis* (NBRC 9182); lane 5, *T. tonsurans* (NBRC 5928); lane 6, *T. interdigitale* (NBRC 5809); lane 7, *T. rubrum* (NBRC 5808); lane 8, *T. violaceum* (CBS 376.92); lane 9, *T. verrucosum* (CBS 562.50). M, 100-bp molecular size marker.

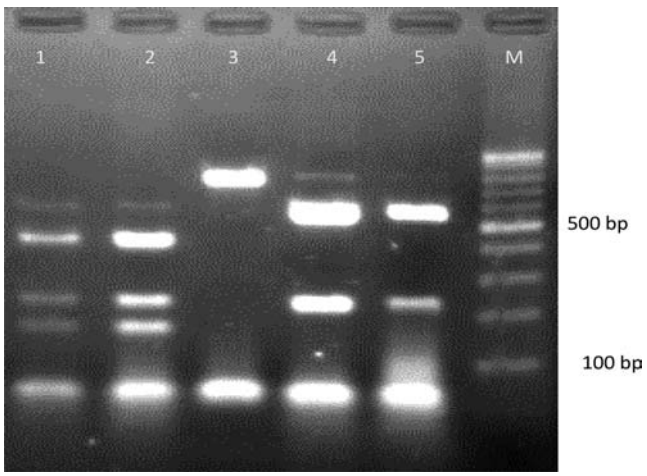

Fig. 2. Restriction maps of BT2 gene by *Alw*21I. Lane 1, *T. interdigitale* (NBRC 5809); lane 2, *T. interdigitale* (NBRC 5466); lane 3, *M. canis* (NBRC 9182); lane 4, *T. tonsurans* (NBRC 5928); lane 5, *T. tonsurans* (NBRC 5945). Contrary to *T. interdigitale* and *T. tonsurans*, the BT2 sequence of *M. canis* has no cutting site for *Alw*21I. M, 100-bp molecular size marker.

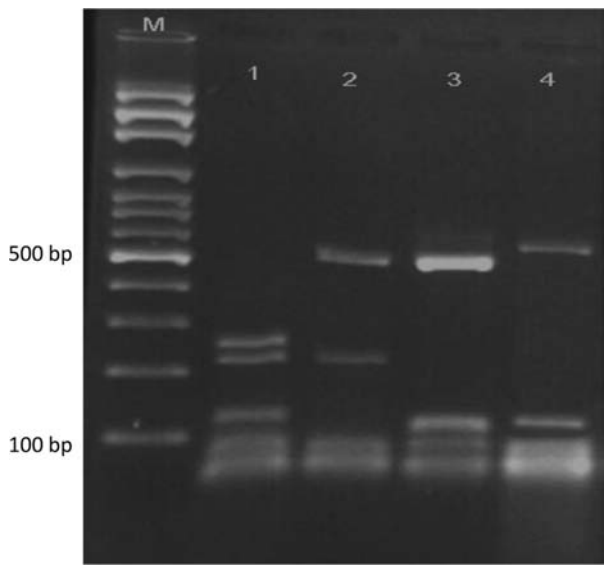

Fig. 3. Restriction patterns of BT2 gene by *Hpy*CH4V for discrimination between *T. schoenleinii* and *E. floccosum*. Lane 1, *T. schoenleinii* (NBRC 8191); lane 2, *E. floccosum* (NBRC 9045); lane 3, *M. gypseum* (CBS 8228), lane 4, *M. canis* (NBRC 9182). M, 100-bp molecular size marker.

DISCUSSION

Phenotypic criteria conventionally used for species identification of dermatophytes are known to be slow and insufficiently discriminative because dermatophytes typically undergo cultural variability (5, 6, 16, 17). Recently, to address these inadequacies, some DNA-based procedures targeting different genetic markers have been used as more rapid and accurate alternatives for dermatophyte identification (6, 8–12). In this study, we developed

a novel and simple RFLP scheme to evaluate the potential and applicable utility of BT2 gene for quick species distinction of the most frequently encountered dermatophytes in Iran (6, 18–21). BT2 protein-encoding gene has been broadly used in fungal phylogenetic analyses because it contains both variable and highly conserved regions (22). It was shown that BT2 digestion provides an easy route for species discrimination among the agriculturally important genera of *Cupressus*, *Ceratocystis,* and *Phaeoacremonium* (23–25), however, there was no similar experience in the realm of medically important fungi. Currently, ITS-rDNA sequencing is the golden standard for species delineation of dermatophytes; however, the method is expensive and may be impracticable for large-scale analysis (16, 17). To overcome these limitations, several comprehensive and sequencing-independent platforms have been developed, in which most of them have used the ITS-rDNA as genetic screening/identification marker (3, 6–8, 26, 27).

Kamiya et al., using combination of a nested-PCR and PCR-RFLP of DNA topoisomerase II gene, developed a protocol for species identification of dermatophytes; however, the introduced procedure exclusively could delineate six species of *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *M. canis*, *M. gypseum*, and *E. floccosum* (28). As exemplified in Figures 1–3 and in accordance with our theoretical analysis, restriction fragment length determination of BT2 revealed that a combination of restriction digestion by *Fat*I, *Alw*21I, and *Hpy*CH4V is useful and applicable for identifying the nine species, *M. gypseum*, *M. canis, T. verrucosum, T. rubrum T. violaceum, T. interdigitale*, *T. tonsurans*, *T. schoenleinii*, and *E. floccosum*. The mentioned species are the predominant agents of dermatophytosis in Iran and worldwide (6, 18–21).

M. canis, *M. ferrugineum*, and *M. audouinii* are three closely-related members of the *Arthroderma otae* complex (16, 18) and it was recently found that interspecies variation of BT2 sequence is higher than ITS for differentiation among the members of *A. otae* complex, however, the two latter species are uncommon species in Iran (18) and we eliminated them in our study. *T. interdigitale* alongside *T. tonsurans* and *T. equinum* comprise three species correlated to *A. vanbreuseghemii* teleomorph (16). The two former species are, respectively, the prevalent agents of tinea pedis and endothrix type of tinea capitis in Iran (6) and were easily distinguished by *Alw*21I in our study but given that human dermatophytosis by *T. equinum* is rare throughout the world, we excluded the species from our restriction analysis. The strains of *T. interdigitale* had intraspecies variations in BT2 sequence, however, surprisingly these variations were not reflected in RFLP and in contrast to Rezaei-Matehkolaei et al. who obtained two ITS-RFLP profiles for *T. interdigitale* isolates (3, 6) we found a unique BT2-RFLP barcode for the species.

The human-adapted dermatophytes, *T. rubrum* and *T. violaceum*, are two closely related species in the *T. rubrum* complex (29). While the former species is the most ubiquitous cause of dermatophytosis worldwide (1, 2, 29) and amongst the common species in Iran, prevalence of the latter fungus has substantially decreased in the country (6, 21). Discrimination between the two species was taken into consideration in some studies. Rezaei-Matehkolaei et al. (3) and Jackson et al. (7) noted that ITS-RFLP with *Mva*I can differentiate these closely related *Trichophyton* species, however, due to the poor resolution of low molecular weight fragments, the observed patterns in gel electrophoresis were not fruitful for this purpose. Conversely, in our investigation the BT2 restriction fragmentation by *Mwo*I was more informative for differentiation of *T. rubrum* and *T. violaceum* especially that a good separation was found by high molecular weight fragments (9, 97, 180, 508 bp for *T. rubrum* vs. 9, 98, 687 bp for *T. violaceum*; Table 1). Owing to some limitations, we did not experimentally perform the discrimination between *T. rubrum* and *T. violaceum* species. But regarding to the fully congruence of the restriction profiles by *Fat*I, *Alw*21I, and *Hpy*CH4V (Figs. 1–3) with the fragments sizes deduced from computational analysis, it is certainly predictable that the estimated fragments sizes of BT2 after digestion with *Mwo*I can be applicable for differentiation of the mentioned species. It was shown that enzymatic digestion of ITS-rDNA regions with *Mva*I generates band patterns characteristic of the anthropophilic species, *E. floccosum* and *T. schoenleinii* (3, 7). Likewise, in the current study, two species were nicely distinguished through BT2 fractionization by *Hpy*CH4V (Table 1, Fig. 3). The geophilic dermatophyte, *M. gypseum,* which is less frequently cited as a source of human glabrous skin and scalp infection (2) and the common zoophilic species, *T. verrucosum* were also distinguishable by BT2 digestion.

Conclusively, given that the species assignment of all studied strains by BT2-RFLP was concordant with ITS-RFLP/ITS-sequencing, the proposed restriction assay would be an affordable, reliable, and relatively rapid screening tool for differentiating the clinically relevant dermatophytes in Iran. The findings of the study emphasize that the BT2, as a new genetic marker other than ITS, has the potency of species differentiation of medically important dermatophytes.

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