A comparative study on morphological versus molecular identification of dermatophyte isolates

Étude comparative pour l’identification des dermatophytes: morphologie versus identification moléculaire

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KEYWORDS
Dermatophytes; Identification; T. rubrum; T. interdigitale; PCR-RFLP

Summary
Objective. — Dermatophytes are taxonomically classified in the genera Trichophyton, Microsporum, and Epidermophyton. Pleomorphism, cultural variability, slow growth and sporulation, and the need for additional physiological tests make dermatophytes notoriously difficult to identify. The present study aimed to compare the results of morphological and molecular identification of certain groups of clinical isolates of dermatophytes with a view to evaluating the accuracy of molecular methods.

Patients and methods. — For each sample, the ITS1-5.8S-ITS2 rDNA region was amplified using the primers ITS1 and ITS4. PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis using the enzyme MvaI and isolate identification was performed by comparing the electrophoretic RFLP patterns with reference profiles obtained previously. Finally, paired comparative analyses of molecular and conventional methods were performed.

Results. — While morphology results from routine daily reports of the laboratories indicated that 18 (6.8%) and 136 (52.10%) of the isolates were T. rubrum and T. interdigitale, respectively, PCR-RFLP results suggested that T. rubrum was the most common etiological agent of ringworm accounting for 94 (36.01%), followed by T. interdigitale accounting for 71 (27.20%). Interestingly, 80.8% of the 94 isolates identified as T. rubrum by molecular testing had been identified by...
morphological examination as belonging to different species, such as *T. interdigitale* (75.5%), *E. floccosum* (2.1%) and *M. canis*, *T. verrucosum*, and *T. tonsurans* (each 1.06%). Ten strains out of 261 (*T. interdigitale*, *n* = 8; *E. floccosum*, *n* = 2) had been defined as unknown species by morphological tests.

**Conclusion.** — An unexpected high percent of isolates identified as *T. interdigitale* by conventional methods were in effect *T. rubrum* shown by PCR-RFLP, and regarding the necessity of correct identification of dermatophytes recovered from different clinical forms of the infection, we highly recommend ITS-sequencing or ITS-RFLP of the isolates, particularly for epidemiological research studies.

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**MOTS CLÉS**
Dermatophytes; Identification; *T. rubrum*; *T. interdigitale*; PCR-RFLP

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**Introduction**

Dermatophytes are fungi which cause infection of the skin, nails, and hair, often spread by air or soil, due to the environmental factors associated with the dermatophytes [7]. The etiologic agents of fungal disease are distributed worldwide and it is estimated that 20%–50% of the total human population is affected by at least one form of dermatophytosis [7]. The distribution of dermatophytes and dermatophytosis in a given geographical area depends on climate, environmental or socio-economic factors, and tourism trends to change over time. The most common dermatophytes in Europe include *Trichophyton rubrum*, *Microsporum canis* and, *Trichophyton interdigitale*; in Asia and Australia, *T. rubrum* and *T. interdigitale*; in Africa, *Microsporum audouinii* and *Trichophyton violaceum* and in the Americas are *T. rubrum*, *Trichophyton tonsurans*, and *T. interdigitale* [13,3].

Correct identification of causative agents of disease is important for epidemiological purposes, control of potential sources of infection, accurate antifungal therapy, prevention of transmission to others and, include exact differentiation between dermatophytosis and non-dermatophyte superficial infections [29,2]. Globally, current baseline dermatophyte species delineation in most laboratories rely on culture-based criteria including micro- and macro-morphology of the colony, mating ability, and, biochemical/physiological characteristics [30]. Over the past two decades, the phylogenetic concept of species strongly revolutionized the taxonomy of dermatophytes, and to ease accurate identification of these fungi, focus has shifted towards culture-independent strategies using molecular methods. These procedures have the advantages of speed, low degree of handling and skill required, and increased sensitivity and reproducibility compared with conventional diagnosis [16]. Apart from the location of study, different frequencies were reported for species in conventional and molecular-based surveys of dermatophytosis [24,31,6].
As previous data have indicated that the DNA sequence-based molecular approach such as sequencing or PCR-RFLP could be more reliable than phenotypic approaches such as morphology assessment of dermatophytes colonies [33,15,12,21,25]. The aim of the current study was to compare results from daily reports of the medical mycology laboratories on species identification of clinical isolates of dermatophytes based on morphological criteria and the results of molecular identification in order to evaluate the relative accuracy of the two diagnostic approaches.

Patients and methods

From September 2012 to September 2013, clinical specimens including nail clippings, subungual debris, and skin scrapings of patients suspected of dermatophytosis, referred to two medical mycology laboratories in Tehran, Iran, were collected. Patient information such as age, gender, and anatomical site of the lesions was recorded. One part of each specimen was routinely subjected to direct microscopic examination for fungal hyphae using potassium hydroxide (KOH 15%) solution, and the other part was inoculated onto mycobiotic agar (Difco, Detroit, MI, USA) and incubated at 27 °C for at least 3 weeks and checked twice weekly for growth. Identification of recovered strains from cultures was done separately by the laboratory technicians based on the typical macroscopic and microscopic features of the colonies, and, if necessary, supplementary tests like urease or hair perforation tests. Subsequently, dermatophyte colonies were submitted to our laboratory for molecular analysis. Hence, morphological identification was routinely performed by the clinical laboratory, and subsequently some of the colonies were collected and processed for molecular identification. In total, 261 strains were referred to our laboratory for DNA-based analysis. The isolates were analyzed by a polymerase chain reaction-restriction enzyme (PCR-RE) method already running in our laboratory [31].

For DNA extraction, a small part (approximately 5 mm in diameter) of the colonies was allocated to a 1.5 mL tube containing 300 μL of lysis buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% w/v SDS, 100 mM NaCl), 300 μL of phenol/chloroform, and 300 μL of glass beads (0.5 mm in diameter). The suspension was vortexed for about 5 min, centrifuged at 5000 rpm for 5 min, and the supernatant was transferred to a new tube and re-extracted using chloroform. The DNA was precipitated with an equal volume of 2-propanol and 0.1 volume of 3 M sodium acetate (pH 5.2), kept at −20 °C for 20 min, and centrifuged at 10,000 rpm for 10 min. The pellet was washed with 300 μL of 70% ethanol, air dried, and finally the DNA was resuspended in 50 μL of sterile, distilled water.

For PCR amplification, ITS1-5.8S-ITS2 rDNA was amplified for each sample by using the universal primers ITS1 (5'-TCCGATTGGAACCTGCGG-3') and ITS4 (5'-TCTCCGGCTTATTGATATGC-3') [35] in a 25 μL reaction mixture, containing 12.5 μL of premix (Ampliqon, Denmark), 2 μL of DNA template, and 0.5 μM of each forward and reverse primer. PCR cycle parameters were as follows: preheating at 94 °C for 6 min; 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C; the final extension time was 10 min at 72 °C. To identify the species, PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis as described previously [30]. Digestion was performed by incubating 8 μL of PCR product with 0.5 μL of the enzyme MvaI fast digest (Fermentas Life Sciences, Lithuania), 1.5 μL of 10X buffer, and 5 μL of water at 37 °C for 20 min. PCR amplicons and digested products were analyzed by agarose gel electrophoresis in TBE (Tris 0.09 M, Boric acid 0.09 M, EDTA 2 mM buffer) at 100 V for approximately 60 min in 1.5% and 2% agarose gels, respectively. For identification of species, the size of fragments generated by enzymatic digestion was compared with profiles obtained previously [30]. Finally, a comparative analysis of molecular and conventional methods was performed.

For sequencing of a single sample, the amplicon was purified using a PCR purification kit (Bioneer, Korea) and sequenced using the ITS1 and ITS4 primers and the BigDye Terminator Cycle sequencing kit by Bioneer (Korea). Verification of species identity was performed with dermatophytes ITS-DNA barcode in the open access NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Results

According to the routine reports of the clinical laboratory and the results of morphology-based methods performed for identification of the dermatophyte isolates, 6.8% (n = 18) and 52.1% (n = 136) of the isolates were identified as T. rubrum and T. interdigitale, respectively. The isolates were also identified by the optimized PCR-RFLP method. Fig. 1A shows an example of restriction profiles of dermatophyte species isolated from the patients. The DNA data showed that T. rubrum with 36.01% (n = 94) cases is the most common etiological agent of ringworm, while T. interdigitale accounted for 27.2% (n = 71) of the strains. Hence, seventy six (80.8%) out of the 94 isolates identified as T. rubrum in molecular tests had been identified by morphological examination as a different species, including T. interdigitale (75.5%, n = 71), Epidermophyton floccosum (2.1%, n = 2), and M. canis, Trichophyton verrucosum and T. tonsurans (1.06% each, n = 1).

Among the isolates identified as T. interdigitale based on morphology, 15.4% (n = 11) were misidentified; among these, 72.7% (n = 8) cases were reported as unknown species. An isolate that had been identified as M. canis on macro/micro colony morphology was identified as Arthroderma benhamiae, confirmed by sequencing of the complete ITS region. The sequence of the strain was deposited in GenBank (accession number: KM502235). Fig. 1B illustrates the colony of this case. Four (7.54%) strains identified as T. interdigitale or unknown species according to conventional methods were delineated as E. floccosum by molecular tests.

Among the 29 strains identified as M. canis by PCR-RFLP, 25 had been recognized as M. canis by culture and the remaining four isolates had been identified as E. floccosum or T. interdigitale.

Of the 13 strains recognized as T. tonsurans by PCR-RFLP, only one isolate had been misidentified as T. interdigitale. A summary of mycological versus molecular species-differentiation of the dermatophytes included in the study is shown in Table 1.
indicated in study, most seen was M. 4.9% according MYCMED-522; + Image 54 to 551.

A. ITS-sequencing. (251, agar électrophorethéque)

B. Colony of a dermatophyte isolated from a hand lesion after 20 days of incubation on mycobiotic agar at 30 °C. The fungus had been identified as M. canis by morphological tests and was subsequently identified as A. benhamiae by ITS-seqencing.

A. Profile électrophorétique des produits ITS-PCR après digestion avec Mval pour l’espèce type de dermatophyte testé dans cette étude. Ligne 1 : les bandes correspondent à 247, 159, 124, 89, 50 et 14 paires de bases (pb), ligne 2 (406, 124, 89, 50 et 14 pb) pour T. interdigitale, ligne 3 (360, 158, 141, 20), 4 (361, 231, 169, 20), 5 (441, 165, 103, 28), 6 (368, 164, 95, 65) et 7 (251, 124, 103, 90, 56, 50, 14 pb) sont A. benhamiae, E. floccosum, M. canis, T. rubrun et T. tonsurans, respectivement. La ligne M est une échelle de marqueur de taille à 100 pb d’ADN. B. Colonie de dermatophyte isolée d’une lésion de la main après 20 jours d’incubation sur mycobiotic agar à 30 °C. Le champignon a été identifié comme M. canis par les tests morphologiques puis comme A. benhamiae par le séquençage ITS.

Table 2 displays a list of the tested dermatophyte strains according to gender and affected site of the body. As indicated in Table 2, tinea pedis and tinea faciei were the most and least frequent clinical forms of infection in the study, respectively. In 95.01% (n = 248) of positive cultures, microscopic examination was also positive, while in only 4.9% (n = 13) of the microscopy-negative samples, culture was positive for a dermatophyte colony. Patient age ranged from 4–85 years, and the highest number of patients was seen in the age groups 30–39 and 20-29 years. Except for M. canis, all species were seen more frequently in men than in women.

Discussion
For preliminary identification of dermatophytes, study of micro/macro morphological features of the colonies is essential. Morphological methods are inexpensive and can be accurate in situations where the examination is performed by skilled technicians, using standardized bench marks to recognize and identify the exact features of the species [20]. Atypical morphology, pleomorphism, requirement for specialized media, prolonged incubation time, failure to sporulate, and confusing results of the phenotypic approach necessitated the development of more simple and reliable

Table 1 Comparative identification of dermatophytes, based on morphology and PCR-RFLP of DNA extracted from cultures.

<table>
<thead>
<tr>
<th>Distribution of isolates according to species as identified by PCR-RFLP</th>
<th>Distribution of isolates according to species as identified by morphological tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. rubrum (n = 94)</td>
<td>T. rubrum M. canis T. interdigitale E. floccosum T. verrucosum T. tonsurans Unknown</td>
</tr>
<tr>
<td>T. interdigitale (n = 71)</td>
<td>18 1 71 2 1 1 —</td>
</tr>
<tr>
<td>A. benhamiae (n = 1)</td>
<td>— — 60 3 — — —</td>
</tr>
<tr>
<td>M. canis (n = 29)</td>
<td>— — 1 — — — —</td>
</tr>
<tr>
<td>E. floccosum (n = 53)</td>
<td>— — 25 2 2 — — —</td>
</tr>
<tr>
<td>T. tonsurans (n = 13)</td>
<td>— — 2 — 49 — — 2</td>
</tr>
<tr>
<td>Total</td>
<td>18 27 136 56 1 13 10</td>
</tr>
</tbody>
</table>

RFLP: restriction fragment length polymorphism; PCR: polymerase chain reaction-restriction.

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Figure 1 A. Electrophoretic patterns of ITS-PCR products after digestion with Mval for representative dermatophyte species tested in this study. Lanes 1 (bands correspond to 247, 159, 124, 89, 50 and 14 bp (1st pattern)) and 2 (406, 124, 89, 50 and 14 bp (2nd pattern)) represent T. interdigitale, lanes 3 (360, 158, 141, 20), 4 (361, 231, 169, 20), 5 (441, 165, 103, 28), 6 (368, 164, 95, 65), and 7 (251, 124, 103, 90, 56, 50, 14 bp) are A. benhamiae, E. floccosum, M. canis, T. rubrun and T. tonsurans, respectively. Lanes M represent 100 bp ladder DNA size markers. B. Colony of a dermatophyte isolated from a hand lesion after 20 days of incubation on mycobiotic agar at 30 °C. The fungus had been identified as M. canis by morphological tests and was subsequently identified as A. benhamiae by ITS-seqencing. The fungi...
methods for identification [5]. Nowadays, different DNA-based techniques, such as species-specific PCR, PCR-RFLP, real-time PCR, sequencing of different regions of the DNA, arbitrarily primed PCR (AP-PCR), nested PCR [25,4,10, 22,26], and various targets such as ribosomal-DNA (rDNA), beta tubulin gene, and mitochondrial DNA (mtDNA), have been used for identification of dermatophytes [15,18,1].

Previous studies have shown that PCR-RFLP targeting the ITS-ribosomal is a rapid, easy-to-perform, inexpensive, and reliable method for differentiation of dermatophytes at species level [15,25]. Therefore, in the present study, we analyzed the electrophoretic patterns of ITS-RFLP in 261 isolates recently recovered from patients having tinea, comparing the obtained data with data generated using colony morphology criteria to evaluate the accuracy of species delineation of the isolates.

A recent study on dermatophytosis indicated that Microsporum ferrugineum and T. equinum are very rare in Tehran [31]; hence, we considered the strains with a Mval RFLP pattern of M. canis/M. ferrugineum or T. tonsurans/ T. equinum as M. canis or T. tonsurans, respectively.

According to our data, dermatophytosis was more prevalent in men (59.92%) than in women (40.07%). Previously, similar observations were reported in other parts of Iran [23,19,8]. This may be related to men having a greater exposure to and contact with other community members via professional activities.

The most interesting finding in this study was the comparison of the results obtained by conventional identification methods with those achieved by the DNA-based method. According to the reports of the mycology laboratory, T. interdigitale was the most frequent (52.1%, n = 136) etiologic agent of dermatophytosis, while as many as 52.2% (n = 71) out of the 136 strains reported as T. interdigitale were in fact T. rubrum as recognized by PCR-RFLP. Therefore, T. rubrum should be considered the most frequent etiologic agent of dermatophytosis. Due to several reasons, differentiation of dermatophyte species, particularly differentiating T. interdigitale from T. rubrum, is challenging: First, both T. rubrum and T. interdigitale may have similar micro morphology on Sabouraud’s dextrose agar medium [9]; second, some isolates of T. rubrum may be similar to T. interdigitale in colony morphology with a cottony texture [27]; third, T. interdigitale usually produces only scattered micro-conidia and do not generate macro-conidia, and so it may resemble those T. rubrum strains that produce orange-yellow or melanoid pigment on media [27]. Some investigators have reported T. rubrum as the most common etiological agent of ring worm in Tehran [24,32], while some others have found that T. interdigitale is the dominant cause of infection [31].

Another issue is the knowledge and perception gap existing in the new species concept of dermatophytes. Until now, the nomenclature of dermatophytes has been unstable; however, the ‘‘neotypification’’ by Gräser et al. [12] recently reformed the nomenclature to comply with available genetic data. The former T. interdigitale sensu lato was recently found to consist of four distinct species that are discernible by several genetic targets [12]; thus insisting on the name ‘‘T. interdigitale’’ for these isolates primarily identified by phenotypic methods is confusing. Gräser et al. [11], in a genetic-based revision of the T. interdigitale complex, rendered a new explanation of this name and reapplied it to imply the former T. interdigitale var. quinckeana and a few genetically concordant taxa, e.g. Trichophyton langeronii and Trichophyton sarkisovii. Phylogenetic studies have correlated the taxon to the Arthroderma simii biological clade, which is separated from Arthroderma vanbreuseghemii and A. benhamiae clades. Hence, at present, T. interdigitale ss. str. is the only valid nomenclatural name Trichophyton mentagrophytes [12]. Among dermatophytes, T. interdigitale is the only taxon that unifies strains of both human and animal-associated lineages, and it shows a high frequency of intragenomic polymorphism in ITS-rDNA regions [15,14]. Based on previous studies [30], T. interdigitale ITS genotypes produce at least two different ITS-RFLP patterns, which are not necessarily correlated with a unique restriction digestion pattern or ecological origin. Thus, it appears that it is not possible to identify correlations with morphological features; nor does it appear possible to differentiate between zoophile and antropophile strains of the T. interdigitale using ITS sequence data.

Among our samples, a culture of sample from hand lesions on mycobiotic agar yielded a yellow mould without

**Table 2** Frequency of different types of dermatophytosis and their causative agents as identified by PCR-RFLP. Percentages are given in parentheses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Clinical type</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tinea faciei</td>
<td>Male</td>
</tr>
<tr>
<td>T. rubrum</td>
<td>63</td>
<td>56</td>
</tr>
<tr>
<td>T. interdigitale</td>
<td>56</td>
<td>51</td>
</tr>
<tr>
<td>A. benhamiae</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>T. tonsurans</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>E. floccosum</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>M. canis</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>127 (48.6)</td>
<td>156</td>
</tr>
</tbody>
</table>

RFLP: restriction fragment length polymorphism; PCR: polymerase chain reaction-restriction.
sporulation and with general morphological characters reminiscent of M. canis (Fig. 1B). In ITS-RFLP analysis, the specific band profile for the taxa in the A. benhamiae complex was observed, and ITS DNA sequencing confirmed the identity of the isolate as T. sp of A. benhamiae. Recently, in molecular epidemiological reports from Iran, several very rare or uncommon dermatophyte species, including Trichophyton eriothrophon, M. ferrugineum, Microsporum fulvum, Trichophyton erinacei, and Trichophyton sp of A. benhamiae, were recovered from clinical samples [31,28]. Clearly, in all of these reports, the mentioned species were misinterpreted as different species by the primary phenotypic identification. Obviously, identification of such dermatophytes which may be important in outbreak investigation or epidemiologic surveys is almost impossible by conventional methods only, and can be obtained only by genotype-based methods.

A more successful morphology-based identification was seen in E. floccosum, M. canis, and T. tonsurans, probably because of more specific morphological features in these species. Nevertheless, as some strains isolated from clinical samples may exhibit atypical morphology or pleomorphism or may not sporulate even after incubation for several weeks on different media [34], such cases are typically reported as unknown species or misidentified. Therefore, accurate identification of such strains by a reliable and standardized method is essential.

Conventional methods based on structure, growth rate, colony pigmentation, and size and shape of conidia can be used to identify dermatophytes, and for this purpose, useful text books and atlases are available [5]; however, atypical and pleomorphic cases and/or lack of ample personal skill may lead to some isolates remaining unidentified. On the other hand, dermatophytosis is not a life-threatening disease, and therefore dermatologists usually manage patients based on preliminary laboratory results (direct examination). As a result, the laboratories do not apply all tests for accurate identification of strains, leading to erroneous results. By using an efficient PCR system, correct identification can be obtained within a working day; however, this requires expert man power and is associated with higher costs.

In conclusion, regarding the necessity of correct identification of dermatophytes recovered from different clinical forms of infection, and according to the problems involving application of morphology-based differentiation of fungal species, we highly recommend ITS-sequencing and/or ITS-RFLP of clinical isolates, particularly for epidemiological research studies and at least in reference laboratories lacking mycological specialization.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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