

# Identification and differentiation of *Trichophyton rubrum* clinical isolates using PCR-RFLP and RAPD methods

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**Abstract** *Trichophyton rubrum* represents the most frequently isolated causative agent of superficial dermatophyte infections. Several genotyping methods have recently been introduced to improve the delineation between pathogenic fungi at both the species and the strain levels. The purpose of this study was to apply selected DNA fingerprinting methods to the identification and strain discrimination of *T. rubrum* clinical isolates. Fifty-seven isolates from as many tinea patients were subjected to species identification by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis and strain differentiation using a randomly amplified polymorphic DNA (RAPD) method, with two primers designated 1 and 6. Using PCR-RFLP, 55 of the isolates studied were confirmed to be *T. rubrum*. Among those, a total of 40 and five distinct profiles were obtained by RAPD with primers 1 and 6, respectively. The combination of profiles from both RAPD assays resulted in 47 genotypes and an overall genotypic diversity rate of 85.4%. A dendrogram analysis performed on the profiles generated by RAPD with primer 1 showed most of the isolates (87.3%) to be genetically

related. PCR-RFLP serves as a rapid and reliable method for the identification of *T. rubrum* species, while the RAPD analysis is rather a disadvantageous tool for *T. rubrum* strain typing.

## Introduction

Dermatophytes comprise a highly specialized group of pathogenic fungi that infect keratinized tissues (skin, hair, and nails) of humans and animals, resulting in dermatophytoses, also referred to as tinea infections. Taxonomically, dermatophytes are classified into three genera: *Epidermophyton*, *Microsporum*, and *Trichophyton*, with the latter being the most complex, containing more than 15 species and several different variants within the species *T. mentagrophytes* [1]. The most frequently observed dermatophyte species worldwide is *T. rubrum*, whose infections usually manifest as tinea pedis and tinea unguium (onychomycosis). The prevalence of *T. rubrum* as a causative agent of onychomycosis is particularly high and exceeds 90% in Europe [2].

The identification of *T. rubrum* by means of conventional laboratory methods may not always be easy or straightforward, since *T. rubrum* exhibits substantial phenotypic variability. Contrastingly, a high degree of homogeneity of the *T. rubrum* genome, as revealed by using several anonymous molecular markers, significantly impedes discrimination at the strain level [3, 4]. Yet, as new molecular typing methods are becoming increasingly available, species determination and strain typing are still being improved.

The aim of this study was to apply some of the recently devised DNA fingerprinting methods to the identification and strain differentiation of *T. rubrum*.

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## Materials and methods

The study included 57 isolates of *T. rubrum* recovered from as many dermatological patients from Lower Silesia, Poland (40), Kraków, Poland (8), and Tübingen, Germany (9).

Clinically, onychomycosis showed the highest number of cases (40 patients; 70.2% of all patients), followed by tinea pedis (12; 21%), tinea corporis (2; 3.5%), tinea cruris (2; 3.5%), and tinea manuum (1; 1.8%).

The isolates were primarily identified as *T. rubrum* on the basis of their phenotypic characteristics, such as colony surface texture, reverse pigmentation, the ability of micro- and macroconidia formation, and urease activity, assessed by standard mycological procedures.

A rapid mini-preparation procedure was used to extract fungal genomic DNA [5]. One  $\mu\text{L}$  of purified DNA (ca. 20 ng) was used for each polymerase chain reaction (PCR) assay.

The identification of *T. rubrum* was achieved by restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region of rDNA, as previously described [6]. The amplified products were digested with *MvnI*, *HinfI*, and, where necessary, with *MvaI* restriction endonucleases (Roche), at a 2-h incubation at 37°C. The resulting restriction fragments were separated electrophoretically on 8% polyacrylamide gels and visualized under UV light after ethidium bromide (Et-Br) staining.

Two primers, designated 1 and 6, as devised by Baeza and Mendes-Giannini [7], were used in two separate randomly amplified polymorphic DNA (RAPD) assays. Amplification products were resolved by electrophoresis using 1.5% agarose gels and were photographed under UV light after Et-Br staining. The gel images were analyzed by the Gel Doc system and Quantity One (Bio-Rad, USA) software. A dendrogram of the RAPD profiles obtained with primer 1 was constructed using Dice's coefficient of similarity and the unweighted pair-group arithmetic averaging (UPGMA) clustering method. An 80% genetic relatedness cutoff was used to define clusters.

## Results

Of the 57 clinical isolates assessed in this study and recognized as *T. rubrum* by conventional identification methods, 55 (96.5%) were further confirmed as *T. rubrum* by means of PCR-RFLP analysis. Using *MvnI*, all of the isolates except for one (841/05) yielded restriction patterns consistent with that of *Trichophyton* taxon. Using *HinfI*, 55 (96.5%) isolates produced fragments whose sizes coincided with those expected for *T. rubrum*. One isolate (899/05) generated a pattern corresponding to either *T. mentagro-*

*phytes* var. *interdigitale* or *T. tonsurans*. The isolate was eventually classified as *T. tonsurans*, based upon *MvaI* restriction analysis. One isolate (841/05) could not be assigned to any of the dermatophyte species, distinguishable by their unique RFLP profiles obtained with *MvnI*, *HinfI*, or *MvaI* enzymes.

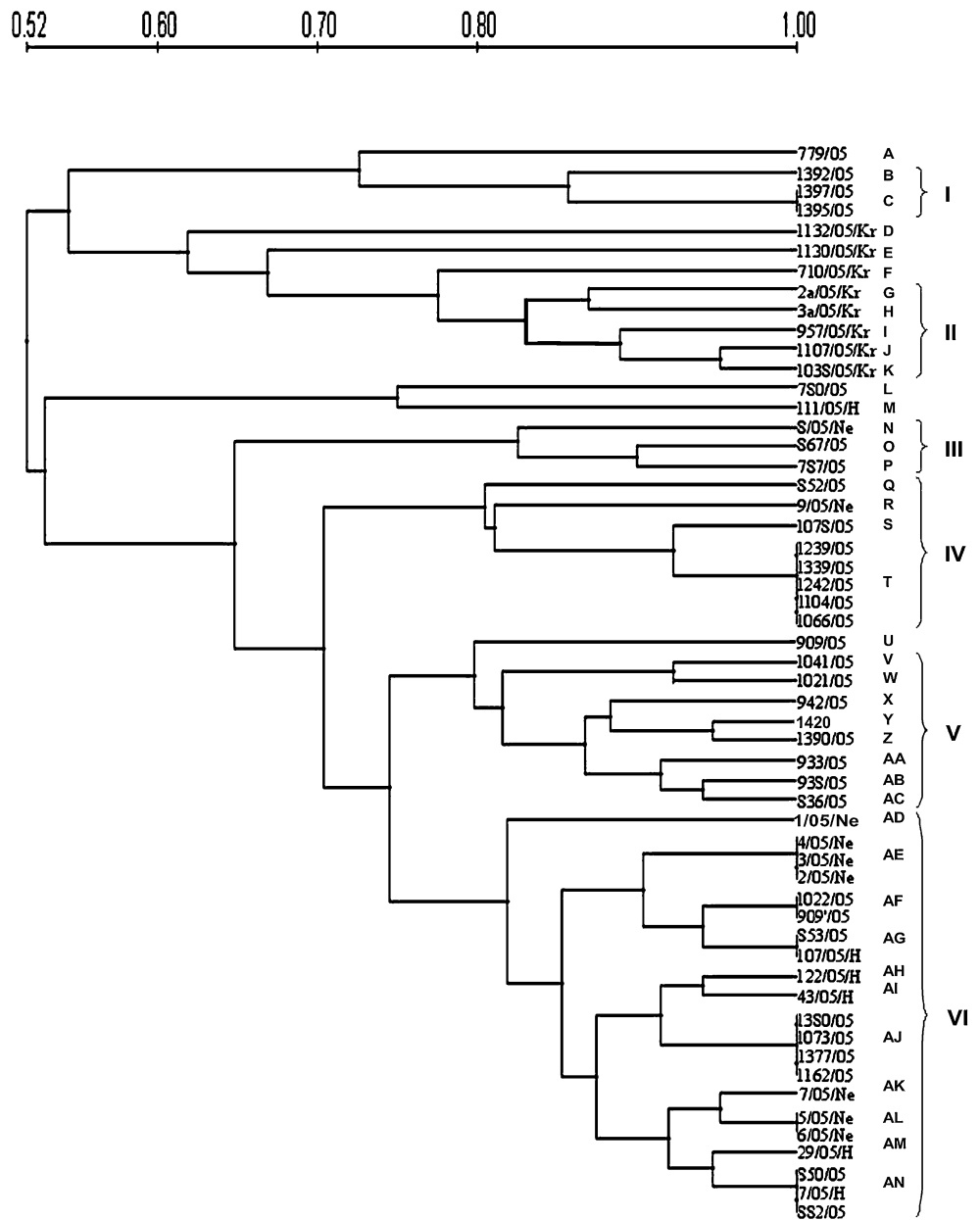
Among the 55 *T. rubrum* isolates, a total of 40 distinct patterns (A–AN) were obtained by RAPD with primer 1. Two additional patterns were observed for non-*T. rubrum* isolates (designated as BB and CC for isolate nos. 841/05 and 899/05, respectively). The four most prevalent patterns, designated as T, AJ, AE, and AN, contained five, four, three, and three isolates, respectively. Four patterns (C, AF, AG, and AL) contained two isolates each, and the remaining 34 patterns were represented by single isolates. The RAPD with primer 6 generated five different profiles in *T. rubrum* isolates (a–e) and two additional profiles in non-*T. rubrum* isolates (designated as “f” and “g” for isolate nos. 841/05 and 899/05, respectively). Among the *T. rubrum* isolates, the most abundant RAPD profile was that designated as “b”, found in 23 isolates, followed by profiles designated as “a” and “c”, represented by 19 and 11 isolates, respectively. Patterns designated as “d” and “e” were each identified for one isolate.

The combination of the profiles obtained with both primers resulted in 47 different genotypes for the *T. rubrum* isolates. Six of those genotypes were common to three (type T-a and type AJ-a) or two (types C-a, AE-b, AF-b, and AN-a) isolates, whereas the remaining 41 genotypes were unique, which are represented by single isolates only.

The distribution of the genotypes varied among the three geographical regions from which the isolates originated. Of the 40 RAPD genotypes produced with primer 1, each was restricted to only one geographical location. Twenty-six genotypes occurred in isolates from Lower Silesia, eight genotypes were observed in isolates from Kraków, and six in isolates from Tübingen, Germany. Regarding the genotypes obtained by RAPD with primer 6, genotypes “b” and “c” were found in isolates from all three regions studied, and genotype “a” was observed in the Lower Silesia and Tübingen isolates, but not in the Kraków isolates. The genotypes “a”, “b”, and “c” were the most prevalent in single regions, i.e., in Lower Silesia (44.7% of isolates), Kraków (62.5%), and Tübingen (44.4%), respectively.

A dendrogram based on the RAPD profiles with primer 1 allowed the separation of the *T. rubrum* isolates into genetic similarity groups (clusters). A total of 48 isolates could be allocated into six main clusters (I–VI), with the similarity index between the isolates within the cluster being 80% or higher (Fig. 1). The largest cluster (VI) comprised 21 isolates, for which 11 different patterns were obtained. Clusters IV and V consisted of eight isolates each,

**Fig. 1** Dendrogram showing genetic relationships among 55 *Trichophyton rubrum* strains inferred from the randomly amplified polymorphic DNA (RAPD) patterns generated by using primer 1



being representative of either four (cluster IV) or eight (cluster V) distinct patterns. The numbers of isolates (and corresponding patterns) belonging to the remaining clusters were 3 (2 patterns) for cluster I, 5 (5) for cluster II, and 3 (3) for cluster III.

**Discussion**

The traditional, culture-based methods of identifying dermatophytes are cumbersome, laborious, and often inconclusive, due to fungal phenotypic variability and pleomorphism [1, 8]. However, the advent of molecular biology tools has enabled the development of new molecular approaches to the diagnosis of dermatophyte

infections. One such approach, introduced by Jackson et al., relies upon RFLP analysis of PCR-amplified ITS regions of the rDNA gene complex [9]. This PCR-RFLP strategy was also employed in the present study. The only modification to the original procedure was the use of *MvnI* and *HinfI* instead of *MvaI*. Whereas digestion with *MvnI* allows discrimination between the three main dermatophyte genera (*Trichophyton*, *Microsporum*, and *Epidermophyton*) [10], the *HinfI* digestion results in species-specific restriction profiles [11]. By using those two restriction enzymes, we wanted to verify their usefulness in the molecular identification of dermatophyte species, and *T. rubrum* in particular. The results from this study showed high concordance between conventional and molecular techniques for identifying *T. rubrum*. Only two isolates were identified as non-*T.*

*rubrum* isolates: one was identified as *T. tonsurans* (upon restriction analysis with *Mva*I) and the other was concluded to be a non-dermatophyte fungus. A possible explanation for the latter may relate to the laboratory contamination of the specimen and/or culture. It is also noteworthy that the application of the PCR-RFLP analysis, as in this study, does not allow to distinguish between *T. rubrum* and *T. soudanense* [9]. The distinction between the two species is only possible with methods that target single nucleotide polymorphisms (SNPs) within the ITS regions of rDNA [12]. Given, however, that *T. rubrum* differs substantially from *T. soudanense* in terms of phenotypic properties and geographical distribution (the former is cosmopolitan, while the latter is restricted mainly to the sub-Saharan part of Africa), the assignment to the *T. rubrum* species provided by PCR-RFLP seems unambiguous.

Differentiation of *T. rubrum* at the strain level has been attempted using a number of genotyping methods, though with unsatisfactory results [3, 13, 14]. However, Jackson et al. have recently reported on intraspecific variability within *T. rubrum* by PCR-amplifying two tandemly repetitive subelements (TRSs), located in the non-transcribed spacer (NTS) region of the rRNA gene cluster [15]. More recently, the RAPD analysis performed by Baeza et al. with two decameric primers, designated 1 and 6, has been shown to produce a high degree of interstrain polymorphism [7, 16]. The RAPD analysis with primers 1 and 6 was also applied in the present study, and this choice was motivated by a high discriminatory potential of the method, higher than that of the TRS typing system [16]. The RAPD typing demonstrated a high genetic diversity of the *T. rubrum* population studied. Based on the combined RAPD profiles, a total of 47 distinct genotypes were obtained. Hence, the overall genetic diversity rate (GDR), calculated as the number of different genotypes divided by the number of isolates, was 85.4%. It is noteworthy that most of the polymorphism was generated by RAPD with primer 1. It yielded 40 profiles, whereas RAPD with primer 6 resulted in only five profiles (GDRs of 72.7% and 9.1%, respectively). In the first study that used primers 1 and 6, among ten clinical isolates of *T. rubrum*, five molecular patterns were observed for each primer [7]. In a subsequent study including 67 *T. rubrum* isolates, a total of 12 and 11 individual patterns were obtained by RAPD with primers 1 and 6, respectively (GDRs of 17.9% and 16.4%, respectively) [16]. Another study that investigated the intraspecific diversity of *T. rubrum* isolates originating from Japan and China revealed a considerable tightness of the population structure. All 150 isolates tested were split into only 19 fingerprinting genotypes, based on the combined RAPD analyses with primers 1 and 6 (GDR of 12.7%) [17]. Much higher genotypic variability with the same primers was shown in a recent study of Santos et al. [18]. Nineteen

different molecular profiles were configured for 52 *T. rubrum* isolates when each of the primers was used independently, resulting in a GDR of 36.5%. It was speculated by the authors that the greater genetic diversity revealed in their study might result from having used strains exclusively from patients with onychomycosis. This condition, with its frequent chronicity, has been associated with mixed infections by multiple *T. rubrum* genotypes [19]. Collectively, the results of the studies cited above differ in terms of genetic polymorphism achieved by RAPD with both of the primers. The polymorphism obtained in our study was exceptionally high, and this may relate to the specific, genetic structure of Polish (and German) isolates, which is different from that of the so-far-analyzed *T. rubrum* populations.

The polymorphism obtained by RAPD with primer 1 was further investigated by a dendrogram analysis derived from the similarity coefficients between the RAPD patterns. Although the similarity coefficient value for the entire *T. rubrum* population studied was calculated to be 52%, a vast majority of isolates (87.3%) were distributed into six clusters, within which all of the isolates shared at least 80% similarity. This finding, considering that the similarity coefficient value of 0.8–0.99 is assumed to represent genetically related isolates [20], indicates that the clustered isolates might have originated from the same strain that had undergone microevolutionary changes [16]. Thus, the high polymorphism resolved by RAPD may relate to the reproducibility of the RAPD technique itself. Indeed, RAPD assays often suffer from poor reproducibility and variations in the amplification patterns between isolates may be caused by even the slightest changes in the PCR reaction conditions. This explains the reluctance on the part of researchers to use the RAPD method and their search for newer molecular tools with a better diagnostic performance. One such promising alternative method is multilocus microsatellite typing (MLMT), which has recently been developed by Gräser et al. [4].

Finally, the fact that every genetic cluster identified in RAPD with primer 1 was restricted to a single geographical locale, together with marked differences in the frequencies of the three most prevalent genotypes produced from RAPD with primer 6, may suggest that certain genotypes exhibit regional and/or geographical affinities. However, since this study was carried out on a relatively small sample of isolates, the geographical specificity of the genotypes would need to be confirmed by further research involving a larger population. Interestingly, the geographical differentiation of *T. rubrum* populations has, so far, been revealed only by using the MLMT methodology [4, 21].

In conclusion, the results from this study demonstrated the usefulness of PCR-RFLP in the rapid identification of *T. rubrum*, yet the insufficient suitability of the RAPD analysis for *T. rubrum* strain differentiation, due to its poor reproducibility.

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