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Trichophyton rubrum and *Trichophyton interdigitale*: Genetic Diversity Among Species and Strains by Random Amplified Polymorphic DNA Method

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Abstract Onychomycosis is a common condition that represents up to 50% of all nail problems and 30% of all cases of dermatophytoses. *Trichophyton rubrum* and *Trichophyton interdigitale* are the most common agents involved in this condition. In cases of recurrent post-treatment onychomycosis, strain fingerprinting could reveal whether the original isolate is responsible, a new strain has been acquired or if multiple strains are involved. The aim of this study was to evaluate the efficacy of the RAPD method for species and strain differentiation of *T. rubrum* and *T. interdigitale* obtained from patients with subungeal distal-lateral onychomycosis. A set of 86 strains of onychomycosis causative dermatophytes were submitted to species differentiation and strain typing by RAPD method with two previously described primers. Both primers proved capable of strain differentiation when tested for each species. Nineteen molecular profiles were configured for *T. rubrum* isolates with primers 1 and 6. For *T. mentagrophytes,* ten molecular profiles were configured with primer 1 and twenty-one with primer 6. We found that *T. interdigitale* and *T. rubrum* species were grouped in different clusters when both primers were analyzed together. This study shows that these primers are valuable tools for strain differentiation with *T. rubrum* and *T. intedigitale.*

Keywords *Trichophyton rubrum* · *Trichophyton interdigitale* · RAPD · Strain typing

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Introduction

Dermatophytosis is among the most common and widespread infectious diseases worldwide [1]. The most common disease agents are cosmopolitan dermatophytes, specifically *Trichophyton rubrum* and *T. interdigitale* (*T. mentagrophytes* var. *interdigitale*) [2, 3]. These conditions are easily treated mycoses, but nail infections (onychomycosis) due to *T. rubrum* are often intractable and more vulnerable to relapse upon cessation of antifungal therapy [4, 5].

The traditional dermatophyte taxonomy is based on gross and microscopic morphology, with minor emphasis on physiology and nutrition aspects [3, 6]. Additionally, dermatophyte phylogeny remains unclear because their members are phylogenetically and taxonomically very closely related, making phenotypic distinctions unclear, and many isolates from medical and veterinary samples have lost sexual activity [7].

With the development of polymerase chain reaction (PCR) technology, several alternative molecular methods have been introduced to type dermatophytic fungi [3, 8]. For example, several markers are

effective for typing dermatophytes: specific amplification of tandemly repetitive elements (TRS) on the non-transcribed spacer (NTS) [4]; restriction analysis of mitochondrial DNA [9]; sequence analysis [7]; restriction fragment length polymorphism (RFLP) analysis of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) [3, 9]; sequence analysis of chitin synthase 1 [10]; and randomly amplified polymorphic DNA (RAPD) [11, 12]. The discrimination achieved by techniques such as mitochondrial DNA restriction analysis is adequate for species identification, but not sensitive enough for strain differentiation [13]. Baeza et al. [14] used two arbitrary primers and detected significant differences between *T. rubrum* isolates. From clinical and epidemiological perspectives, a reliable method for identifying dermatophyte species at the strain level is critical for the prevention and treatment of dermatophytosis [7, 14].

In this study, we have attempted to evaluate the efficacy of the RAPD method for species and strain differentiation of *T. rubrum* and *T. interdigitale* obtained from patients with subungeal distal-lateral onychomycosis.

Materials and Methods

Strains

Eighty-six dermatophyte isolates (52 *T. rubrum* and 34 *T. interdigitale*) were used in this study. They were included also in previous works focused on drug susceptibility and amplification of TRS/NTS regions [15–17]. All of them were obtained from patients suffering of toenail subungeal distal-lateral onychomycosis in Belo Horizonte, Minas Gerais, Brazil. The nail samples were collected, and a single colony was obtained from each sample. These were identified by routine mycological, culture and biochemical analysis, and they were maintained in sterile saline (0.9%) at 4°C and on Sabouraud Dextrose Agar. Forty *T. rubrum* strains were obtained from 20 patients (from A to T) before and after oral therapy over the course of 4 months, with isolates from the same patient (for example, patient A) denominated as A1 (before therapy) and A2 (after therapy). The other *T. rubrum* isolates were designated with “Tr”, and other *T. interdigitale* isolates with “Tm”. The Ethics Committee of Universidade Federal de Minas Gerais approved this work (document ETIC 110/05).

Fungal DNA Isolation

Two milligram of hyphal growth (obtained from a 7-day-old culture) was placed in a 2.0-ml microcentrifuge tube, and 300 μl of lysis buffer (20 mM Tris–HCl [pH 8.0], 0.25 mM EDTA, 250 mM NaCl, 1% SDS, 50 μg/ml Proteinase K) were added. Two freeze-boil cycles at −80°C were carried out. The sample was vortexed for 3 min and incubated for 1 h at 60°C. The mixture was treated with 3 μl of RNase and incubated at 37°C for 15 min. Nucleic acids were extracted first with phenol–chloroform–isoamyl alcohol (25:24:1) and second with chloroform–isoamyl alcohol (24:1). This was followed by precipitation with isopropanol, washing with 70% ethanol and resuspension in 30 μl of 10 mM of Tris–NaCl–EDTA (TE).

RAPD Assays

Two decamer oligonucleotides (sequence 1: 5′-GGT GCGGGAA-3′ and sequence 6: 5′-CCCGTCAGCA-3′) previously described by Baeza et al. [14] were used in RAPD assays. For the reactions, a total volume of 100 μl containing reaction buffer (30 mM KCl, 10 mM Tris–Cl [pH 9.0], 0.1% Triton X-100), 3 mM magnesium chloride, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 2.5 μg of bovine serum albumin (BSA), 25 pmol primer, 5 U of *Taq* polymerase (Phoneutria™, Belo Horizonte, Brazil) and approximately 50 ng of the diluted template DNA. The final volume was made up with pure water. PCR was performed in thermal cycle model PTC 100 (MJ Research, Inc.) as follows: an initial denaturation for 5 min at 95°C was followed by 45 cycles of primer annealing at 36°C for 1 min, extension at 72°C for 2 min and denaturation at 95°C for 1 min. A terminal extension step of 72°C for 10 min completed the reaction. Amplification products were separated by electrophoresis in 2% agarose gels, visualized by staining with ethidium bromide and photographed under UV light. All the tests were repeated twice, and the same results were obtained. RAPD results were analyzed by using LabImage software to compare the number and size of DNA bands amplified from the various *Trichophyton* strains. The dendrograms were obtained by the

Unweight Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm based on the data matrix constructed with the combined results of both PCR methods and built using the TFPGA (Tools for Population Genetic Analyses) computer package program. Dendrongrams were constructed for each primer with each species and for both primers combined with both species. Similarity was graduated from 0 to 100% based on the dendrogram scale.

All the experiments were repeated twice, and the same results were found.

Results

All tested isolates produced distinct banding patterns with both primers, which allowed for differentiating between the isolates. RAPD profiles were assigned according to major bands. All visible and well-defined bands were identified visually and confirmed by software (LabImage). It is worth noting that the age of the lesions differed when fungi were sampled: *T. rubrum* isolates were obtained from lesions ranging from 1 month to 4 years of evolution (average of 2.5 years), while *T. interdigitale* strains were obtained from lesions no older than 7 months (average of 4.3 months). The cluster analysis grouped similar isolates with similar RAPD profiles.

Figure 1 shows the results of RAPD for the 52 *T. rubrum* isolates using primers 1 (Fig. 1A) and 6 (Fig. 1B). In general, we noted few bands in most of the strains tested with primer 1, but bands greater than 1,000 bp were present in most of these strains. The analysis of Fig. 1A allowed us to configure 19 molecular profiles. The isolates grouped in two distinct clusters (1 and 2) with low degrees of similarity (6%). Eight isolates (F1, F2, R2, D2, B1, Tr12, Tr11 and Tr3) showed identical patterns, and these were grouped in cluster 1. All of them showed two major bands near 1,400 bp, with weak bands present above and below this value. Except for F1, B1 and Tr13, the isolates were obtained from lesions with up to 3 years of evolution. Cluster 2 was divided in two subgroups (2A and 2B) with 63% similarity. The subgroup 2A comprised of 23 strains with two major clusters showing high similarity degree and presenting major bands near 2,000 and/or 1,400 bp. The 2B subgroup comprised of 21 isolates presenting bands higher than 2,000 bp and/or lower than 1,000 bp. Intriguingly, this primer grouped isolates from ten patients (C, T, S, N, M, E, F, P, Q and J), obtained both before and after antifungal oral therapy, in clusters with high degrees of similarity. Meanwhile, isolates from other patients (A, B, D, G, H, I, K, L and O) showed distinct RAPD profiles before and after antifungal therapy.

A total of 19 molecular patterns were obtained with primer 6 (Fig. 1B). Primer 6 yielded more bands than primer 1 for *T. rubrum* isolates, which indicates primer 6 better reveals polymorphic regions. We noted that the strain A2 (at the bottom of the dendrogram), which exhibited bands between 800 and 3,000 bp, demonstrated low similarity (4%) with the other tested isolates. Regarding the rest of the strains, we observed two clusters (1 and 2), with cluster 1 constituting 10 isolates presenting major bands between 1,000 and 1,650 bp and a common band at 1,650 bp. Cluster 2 represented the majority of the tested isolates, all of which shared a band near 2,000 bp. When comparing strains obtained at different moments of antifungal oral therapy, primer 6 clustered together isolates from six patients (T, S, F, M, O and J), with five of them (T, S, F, M and J) the same as those clustered with primer 1. Interestingly, isolates from cluster 1 using primer 1 were not identical when analyzed with primer 6.

Figure 2 shows dendrograms obtained for *T. interdigitale* analysis by primers 1 (Fig. 2A) and 6 (Fig. 2B). Ten molecular profiles were revealed when primer 1 was used, all presenting a common band near 500 bp. A total of two major groups were visualized, labeled group 1 and group 2. Group 1 contained 14 isolates in three major clusters, each one showing clonality. All of the strains contained a band near 300 bp, and many had a band near 400 bp and another higher than 1,200 bp. Group 2 was subgrouped in 2A (which contained four different clusters and all isolates exhibiting bands near 1,650 and 1,000 bp but lacking a 400 bp band) and 2B (with three strains, all of them showing bands near 500, 700 and 1,500 bp). Primer 6 allowed us to configure twenty-one molecular profiles within three major clusters (Fig. 2B), all of them containing a common band near 500 bp but lacking a 400 bp band. Group 1 was represented by two isolates (Tm23 and Tm25) showing only 10% similarity with each other and the others, and this group contained numerous bands between 1,200 and 500 bp. Group 2 was constituted by three strains with a common band

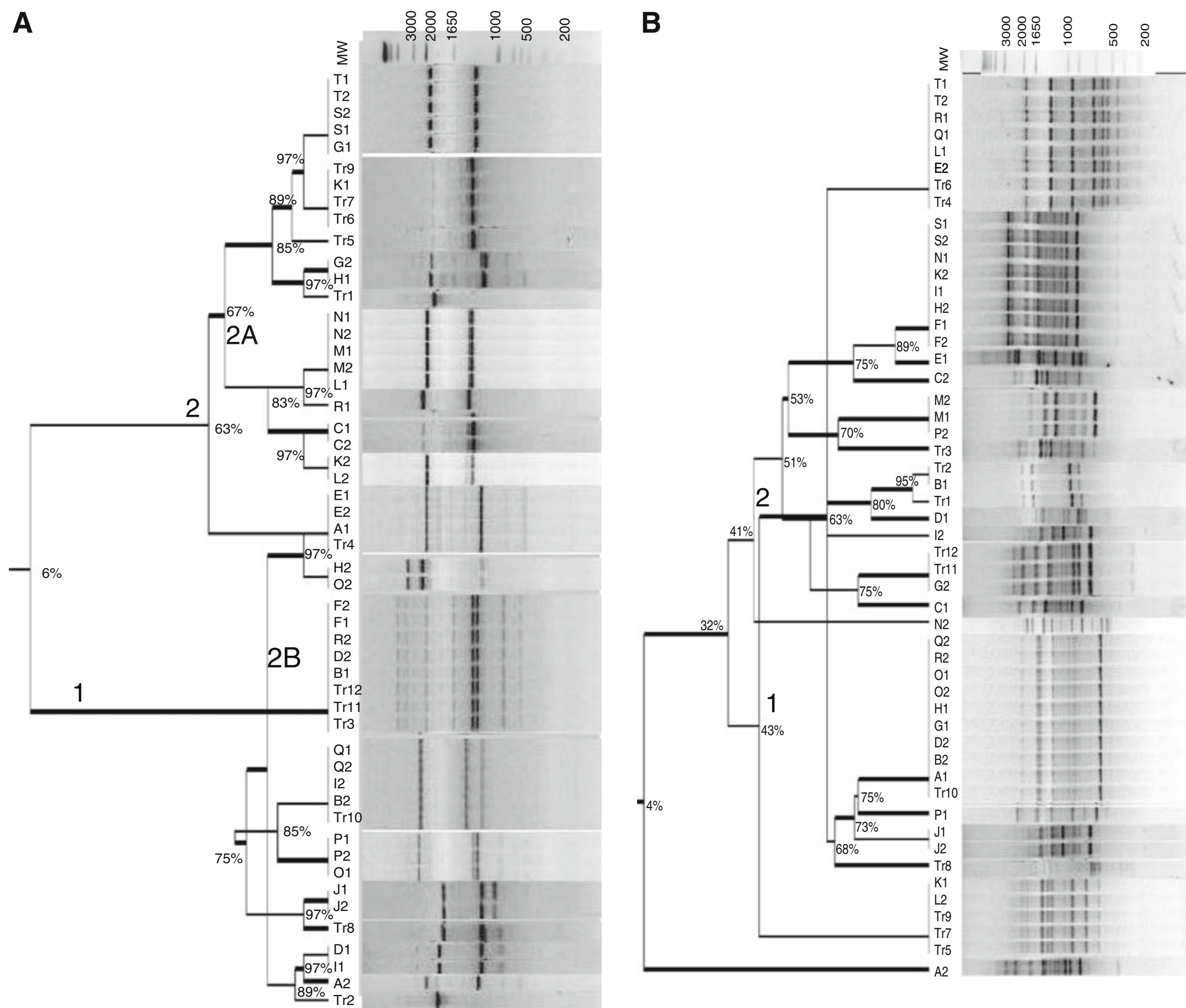

Fig. 1 Dendrogram of the RAPD patterns obtained from 52 *T. rubrum* isolates from onychomycosis patients using primers 1 (**A**) and 6 (**B**). *MW*: molecular weight

near 1,650 bp and with low similarity to the others (22%). Group 3 contained the majority of the strains, which were subgrouped in 3A (18 isolates) with two clusters containing many highly similar isolates. This group was characterized by a common band near 1,300 bp and the absence of bands higher than 1,650 bp. The subgroup 3B, with 9 strains, had an isolate (Tm27) showing 45% similarity with the others, and the rest of the strains grouped in three clusters exhibiting clonality. A common band near 1,200 bp was observed for all the isolates from this group. Strains Tm12 and Tm21 showed low similarity (27%) to other isolates from this group. Primer 6 was also more discriminative for *T. interdigitale* isolates.

Figure 3 represents a global dendrogram of RAPD analysis from the two tested species, using both primers. Four major clusters were observed: the first cluster constituted all eight *T. rubrum* isolates that were previously located in cluster 1 using primer 1 (Fig. 1A), with only 10% similarity with the rest of tested isolates. Five isolates comprised of cluster 2, which corresponds to cluster 1 from primer 6 for *T. rubrum* (Fig. 1B), with 21% similarity to the other groups. Clusters 1 and 2 corresponded to two different groups of *T. rubrum*, each with distinct banding patterns. The majority of the isolates were contained in clusters 3 and 4, which had 33% similarity and contained *T. interdigitale (*cluster 3)

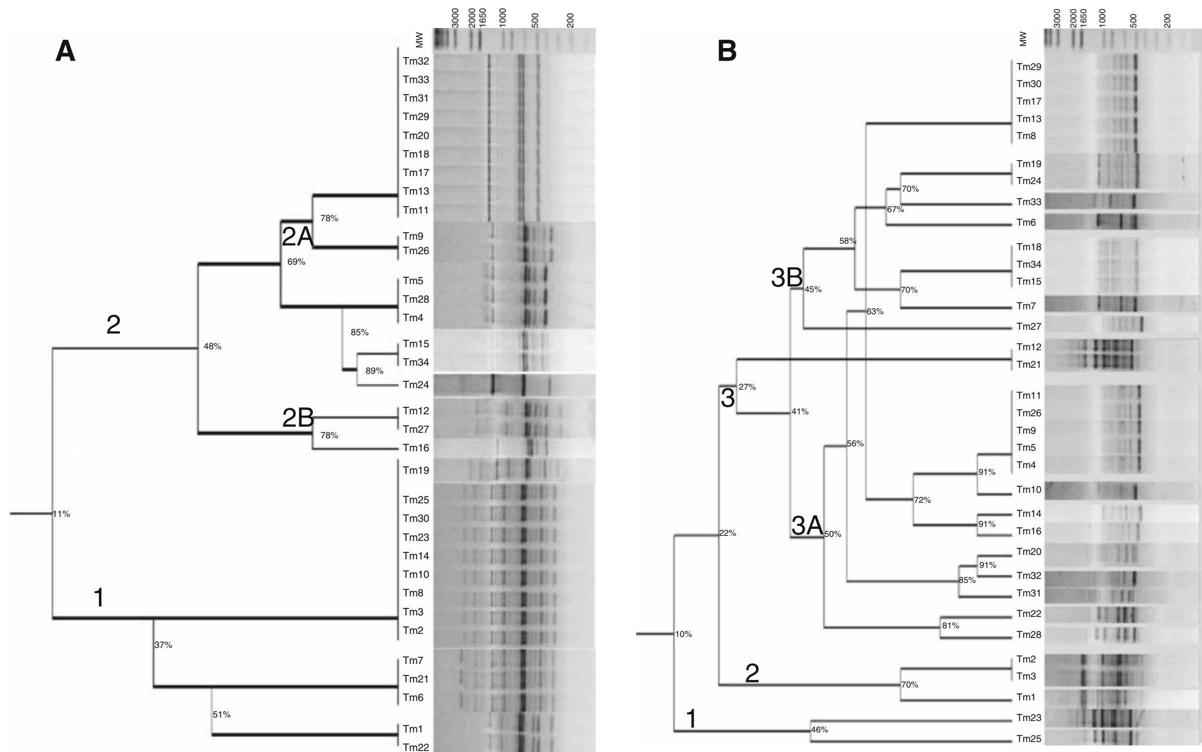

Fig. 2 Dendrogram of the RAPD patterns obtained from 34 *T. interdigitale* isolates from onychomycosis patients using primers 1 (**A**) and 6 (**B**). *MW*: molecular weight

and *T. rubrum* (cluster 4) strains. An exception to this was the *T. rubrum* isolate A2 (arrow on Fig. 3), which grouped on cluster 3 with *T. interdigitale* isolates. Cluster 3 (containing 35 isolates) was subgrouped in 3A and 3B with 37% similarity. By contrast, cluster 4 was composed of 38 *T. rubrum* strains subgrouped in two clusters with 43% similarity (4A and 4B). The first one (4A) contained 20 isolates derived from different timings of antifungal therapy of the patients T, S, M and J and grouped by this way using both primers. Eighteen strains grouped in cluster 4B, being isolates from patient F with the same RAPD profile. *T. rubrum* isolates from patients D, L and R (D2 and R2 are in the cluster 1 and L2 is in the cluster 2) obtained before versus after antifungal therapy were distinct, with "before therapy" isolates grouping in cluster 4. Aiming the comparison between different typing methods, this figure also presents the TRS–NTS profile obtained in the previous work (17) for the strains of *T. rubrum*. In that work (17), five molecular profiles were obtained when the TRS-1 subunit was analyzed (1–5) and three when TRS-2 was tested (I–III).

Discussion

Dermatophytosis is one of the most frequently observed conditions in biomedicine, but the approximately 25 pathogenic species identified as the causative agents of this condition lack clear taxonomic relationships [8]. However, the recent advent of molecular biological techniques has enabled the detailed examination of dermatophyte species structure and evolution [18]. One of the applications of a molecular typing method is establishing the relatedness of isolates collected from different patients or from the same patient [19]. For this purpose, RAPD has a number of advantages over other molecular techniques, including small quantities of DNA template required, simplicity and speed of execution, relatively low cost, and the scope to handle many

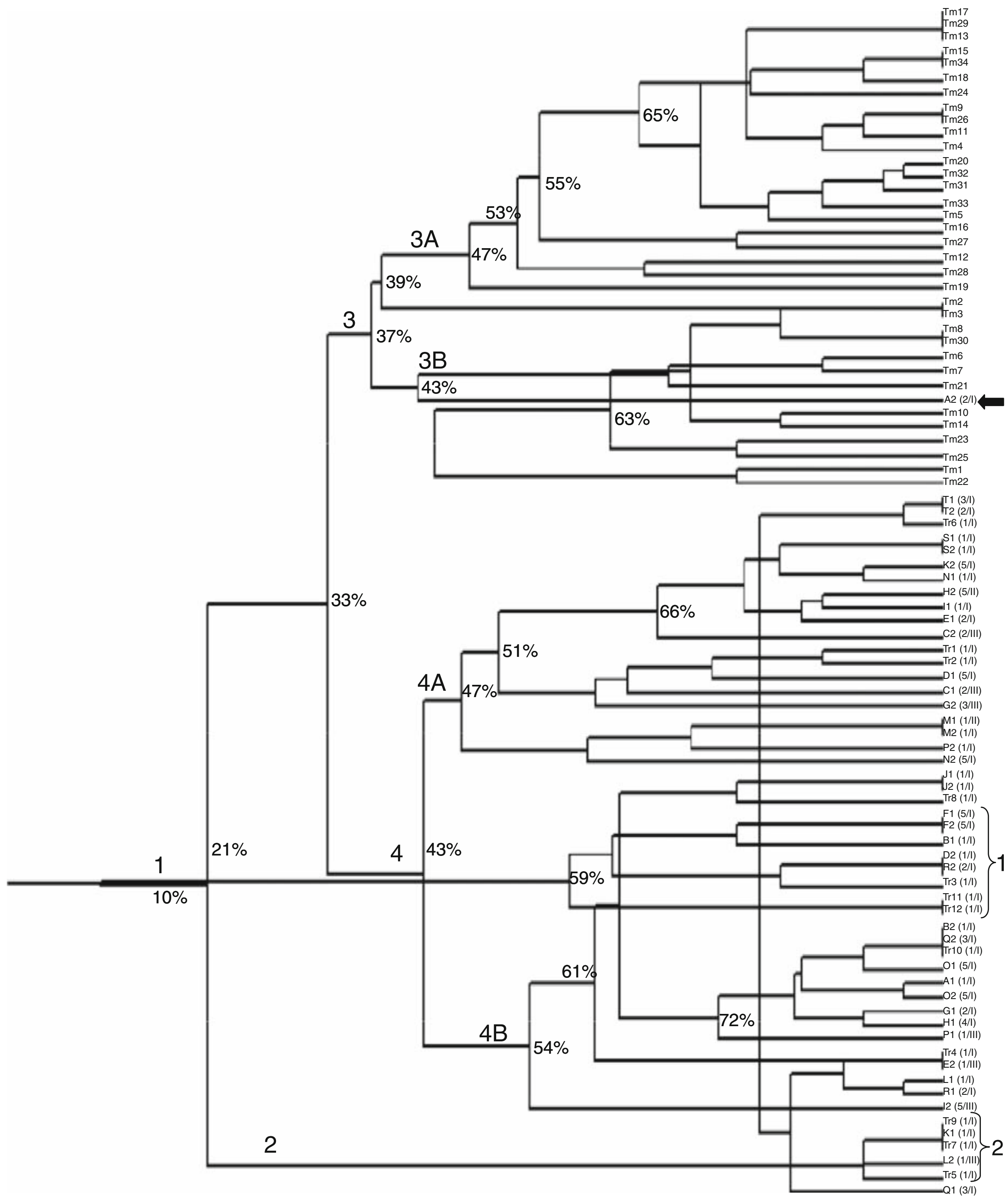

Fig. 3 Dendrogram of the RAPD patterns obtained from 52 *T. rubrum* and 34 *T. interdigitale* isolates from onychomycosis patients using primers 1 and 6. Arrow points to *T. rubrum* isolate A2. The TRS type for the *T. rubrum* strains are presented in *parenthesis* with TRS-1 type (1–5) followed by the TRS-2 profile (I–III) obtained in previous work (17)

polymorphic loci randomly sampled across whole genomes [20].

Our results show that band profiles for *T. rubrum* isolates were generally simpler than those from *T. interdigitale*. The latter contained more bands lower than 500 bp, while bands near 2,000 and 3,000 bp were common in *T. rubrum* strains. Both primers demonstrated individual discriminative abilities for the two tested species, and higher clonality was found when primer 1 was used. The dendrogram obtained with both primers combined (Fig. 3) provided more analytical information than the others (Figs. 1 and 2) because it factors in differences among species and strains intrinsic to each primer.

Techniques for the detection of intraspecific variation are sought for population genetic studies in *Trichophyton* species, especially *T. rubrum*, an anthropophilic dermatophyte exhibiting unusually high degrees of uniformity [2]. Recently, the detection of intraspecific variation in *T. rubrum* was reported using PCR amplification of tandemly repetitive elements (TRSs), TRS-1 and TRS-2, from the rDNA non-transcribed spacer region, demonstrating that this technique is suitable for differentiating between isolates [4, 17, 21]. Baeza and Mendes-Giannini [12] used primers 1 and 6 to type 10 *T. rubrum* isolates, and they found five molecular profiles for each primer. In 2006, Baeza et al. [14] performed the same typing method for 67 *T. rubrum* strains and found 12 molecular types with primer 1 and 11 with primer 6. That study [14] used isolates from several kinds of tinea lesions (onychomycosis, *tinea pedis*, *tinea manuum*, *tinea cruris* and *tinea corporis*), while our study only used strains provided from onychomycosis patients. We obtained 19 molecular profiles with primer 1 and 19 with primer 6, with higher polymorphic diversity when all isolates were obtained from only one kind of lesion. Yazdanparast et al. [21] demonstrated that multiple strains of *T. rubrum* can exist in a single affected nail. Lower degrees of *T. rubrum* polymorphism were observed from lesions of *tinea pedis* [22] and *tinea corporis* [23]. Yang et al. [24], in a recent work, tested 150 *T. rubrum* strains obtained from Japanese and Chinese patients using the same primers used here. The RAPD method was compared to the sequence of the NTS region of the rDNA gene. The authors suggested that the origin of the isolates from these countries may not be identical.

The greater genetic diversity revealed in our study may result from using strains from onychomycosis patients, who tend to manifest lesions with major chronic characteristics independent of geographic correlations. It is worth noting that the evolution in *T. rubrum* populations occurs through geographic isolation, which results in a limited range of ecological niches due to the presence of competing species. This corroborates the observation that the mixed infection by strains with identical molecular profiles is uncommon [8], especially considering chronic disease evolution. According to previous works [17, 21], differences between the molecular profiles obtained from *T. rubrum* isolates before and after the antifungal therapy of onychomycosis might be due to the existence of multiple strains of this dermatophyte in only one affected nail or the patients may soon acquire another strain from the environment.

Gräser et al. [8] noted that *T. rubrum* population structuring might account for their predilection on the human host, which can perpetuate (micro) climatic differences depending on the body sites of infection, being the prominent genotypes found the one that better adapt to the environmental conditions. This fact may partially explain the discrepancy of clusters 1 and 2 from Fig. 3 (also seen on Fig. 1A, b), which comprise *T. rubrum* strains with low similarity (10 and 21% for clusters 1 and 2 in Fig. 3, respectively) with the other *T. rubrum*, which causes chronic lesions with several years of evolution, promoting different environmental conditions over that time, which influences strain(s) selection during diagnosis.

This is the first report using primers 1 and 6 for the molecular typing of *T. interdigitale*, which yielded 10 and 21 molecular profiles, respectively. Previous studies have focused only on *T. rubrum* [14]. Detailed understanding of *T. interdigitale* transmission, as well as insights into its pathogenic mechanisms, requires a method for distinguishing between strains [9]. Kim et al. [11] performed RAPD analysis of 61 *T. interdigitale* isolates and concluded that this was a viable technique for subtyping this species. Recently, restriction fragment length polymorphism (RFLP) analysis of the NTS region of the rDNA was reported as a robust technique for typing *T. interdigitale* [9]. Kaç et al. [19] performed *T. interdigitale* differentiation by RAPD for 46 strains provided from patients from different continents. That study found no correlation between RAPD profiles and continental

origins of isolates. Of all isolates tested by Kaç et al., only seven were from toenail onychomycosis patients, and these strains showed low similarities. Our data demonstrated that primers 1 and 6 are useful for the *T. interdigitale* strain typing.

We observed that *T. rubrum* isolates from patients T, S, F, M and J demonstrated higher similarity based on analyses using both primers, which indicates that strain RAPD profiles were consistent before and after antifungal oral therapy. These isolates were previously typed by the amplification of TRS-1 and TRS-2 repetitive elements from the ribosomal DNA non-transcribed spacer regions. In that study, patients F, S and J provided isolates with the same molecular profile before and after antifungal oral therapy (1/I, 1/I and 5/I, respectively) [17]. Different results were previously found for patients T and M by TRS analysis, which yielded two distinct strains on two different clinical examinations. The 1/I TRS profile was predominant in all the *T. rubrum* clusters on Fig. 3, corresponding to 62.5% of the cluster 1; 80% of the cluster 2; 45% of the cluster 4A, and 45% of the 4B. We might expect different results using distinct techniques for typing our strains. First of all, TRS amplification is specific to the NTS regions of DNA, while RAPD provides information from the entire genome of the fungi, which likely exhibits more polymorphic regions compared to the NTS. Furthermore, the two techniques provided analytical information regarding the *T. rubrum* strain typing, with each one representing one way to present data. On the other hand, we think that further studies involving dermatophyte isolates from patients using antifungal drugs are still necessary to establish the predictive value of RAPD with primers 1 and 6 and the TRS–NTS typing methods. In addition, dendrograms constructed using both primers on strains of both species yielded results with more interpretative meaning. Isolate A2 grouped with *T. interdigitale* strains, making its real identity suspect, but it was previously submitted to PCR for the NTS regions, which is considered a specific technique [4]. Nucleotides sequence methods might confirm this data. This strain provided the 2/I profile in the TRS analyzing, and this was also noted for the isolates E1, G1, R1, R2 and T1 [17]; all of them were distributed in distinct clusters in the global dendrogram (Fig. 3). In conclusion, we have demonstrated that primers 1 and 6 are potentially valuable tools for molecular-level discrimination between *T. rubrum* and *T. interdigitale* and for typing different strains within each species.

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