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BRIEF COMMUNICATION

STRAIN DIFFERENTIATION OF *Trichophyton rubrum* BY RANDOM AMPLIFICATION OF POLYMORPHIC DNA (RAPD)

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SUMMARY

Trichophyton rubrum is an important cause of dermatomycoses. Molecular strain typing methods have recently been developed to address questions about epidemiology and source of relapse following treatment. This report describes the application of RAPD for molecular strain differentiation of this fungus utilizing the primers 1- (5'-d[GGTGCGGGAA]-3') and 6- (5'-d[CCCGTCAGCA]-3'). A total of five RAPD patterns were observed among 10 strains of *T. rubrum*, with each of the primers used. We conclude that RAPD analysis using primers 1 and 6 can be used in epidemiological studies.

KEYWORDS: *Trichophyton rubrum;* Molecular typing; RAPD.

Dermatophytes are keratinophilic fungi responsible for causing dermatophytosis in man and animals[13]. *T. rubrum* is an important etiological agent of dermatomycoses, principally causing tinea pedis and tinea unguium.

In the last few years, genotypic approaches have proved to be useful for solving taxonomic problems regarding dermatophytes[8] . Molecular typing could be of great use in epidemiological questions, e.g. routes of infection, common sources of infection, and dissemination areas as well as to determine whether the original isolate is responsible for relapse (i.e., treatment failure) or reinfection has occurred with a new isolate. Strain identification in the *T. rubrum* has been attempted using a range of molecular-typing procedures[2,8,15]. But until now, molecular genotyping of this fungus has limited success[2,9] and the extent of interstrain genomic variation within this apparently clonal species appears to be limited[2]. Recently, the detection of intraspecific variation in *T. rubrum* was reported by using amplification of subrepeat elements (TRS-1) from the ribosomal DNA nontranscribed-spacer region (NTS), with 21 TRS-1 PCR types recognized from 101 clinical isolates[4], as well as, KAMIYA *et al.*[6] observed 17 TRS-1 PCR types in 252 clinical isolates. YAZDANPARAST *et al.*[14] also reported two or more *T. rubrum* strain types from an infectious site when these strains were analyzed by PCR-based typing method with variations in numbers of repetitive elements in the NTS of the ribosomal RNA gene repeats. In practic, RAPD could distinguish *T. rubrum, T. mentagrophytes,* and *T. tonsurans*[8], whereas intraspecific variability has only been observed in the *T. mentagrophytes* group[5]. The aim of the present study was to investigate genotypic variability within *T. rubrum*, using RAPD with six random primers.

Strains: A total of 10 strains were isolated from patients with onychomycoses, in the Clinical Mycology Laboratory of the Faculty of Pharmaceutical Sciences, University of São Paulo State (UNESP), Araraquara, SP, Brazil, and identified to species level using standard mycological procedures[11].

Isolation of fungal DNA: All isolates were grown on Sabouraud's dextrose agar (Difco) supplemented with chloramphenicol 0.05% [w/v] and incubated at 25 ºC for 10 days. Liquid nitrogen was added to 1 to 2 g of mycelia in a mortar, and the cells were grounded with a pestle. Genomic DNA was extracted as described by DEL SAL *et al.*[1] with a few modifications. In brief, the powdered mycelium was transferred to an Eppendorf tube and 500 µL of lysis buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 1% sodium dodecyl sulfate [SDS]; 100 µg mL^{-1} proteinase K) were added. Each sample was incubated for one h at 37 ºC, and then 200 µL of 5-mol L^{-1} NaCl were added and incubated for 10 min at 65 ºC. After that, 100 µL of 10% [w/v] cetyltrimethylammonium bromide (CTAB; Sigma) were added and incubation continued for a further 20 min at 65 ºC. The solution was treated with RNase A (Roche) at a final concentration of 50 µg mL^{-1} for one h at 37 ºC, and then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1[v/v]), and centrifuged at

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12,000 g for 15 min at 4 ºC. This procedure was repeated three times. The DNA was precipitated with two volumes of ice-cold isopropanol at -20 ºC for 20 min, washed twice in 500 µL of 70% ethanol, air dried, and resuspended in 100 µL of TE buffer (40 mM Tris-HCl, pH 8.0; 2 mM EDTA). DNA concentration was estimated by measuring the optical density at 260 nm.

RAPD: The following oligonucleotides (decamers) of arbitrary sequence were used as single primers in the RAPD experiments: 1-(5'-d[GGTGCGGGAA]-3'), 2- (5'-d[GTTTCGCTCC]-3'), 3- (5'-d[GTAGACCCGT]-3'), 4- (5'-d[AAGAGCCCGT]-3'), 5-(5'-d[AACGCGCAAC]-3'), 6- (5'-d[CCCGTCAGCA]-3'). Amplification reactions were performed in volumes of 25 µL containing 50 ng of template DNA, and a lyophilized mixture of a reaction buffer [30 mM KCl; 3 mM $MgCl_2$; 10 mM Tris (pH 8.3)], 0.4 mM of each dNTP, BSA 2.5 µg, 25 pmol of primer, and thermostable polymerases (Ampli Taq™ DNA polymerase and Stoffel fragment) (Ready-To-Go™ RAPD Analysis Beads- Amersham Pharmacia Biotech). The samples were overlaid with sterile light mineral oil (Sigma) and amplification was performed in a thermo cycler (Perkin-Elmer 9700) as follows: one initial cycle for five min at 95 ºC, followed by 45 cycles comprising denaturation (one min at 95 ºC), annealing (one min at 36 ºC); and extension (two min at 72 ºC) and then a final extension for 10 min at 72 ºC. Amplification products were separated by electrophoresis in 2% agarose gels, visualized by staining with ethidium bromide, and photographed under UV.

Two out of the six different random decamers examined (primers 1 and 6) reflected intraspecific polymorphisms within *T. rubrum*. Primer 1 yielded five different patterns among the isolates. The number of fragments varied from five to 12 bands ranging between approximately 1,800 and 180 bp in length. The isolates presented two common bands at 180 and 280 bp. Some groups of patterns (patterns A and B; D and E) were similar; in patterns A and B only two of the bands were not common (1,800 and 1,100 bp). Profile D showed a strong fragment at 400 bp, which was absent in profile E. Pattern C was the most different, and had only a few bands common with any of the other patterns (Fig. 1).

As shown in Fig. 2, five RAPD types were also observed among the strains studied with primer 6. The number of fragments varied from three to eight bands, ranging between approximately 1,300 and 200 bp in length. The RAPD profiles had two broad bands at 450 and 650 bp, which were common among all isolates. Profile A was the most common, representing four (40%) of the 10 strains. Profile type B showed one fragment of 200 bp in addition to those in profile A, type C presented two bands (200 and 550 bp) different of the pattern A. Profile type D showed four fragments (1,300, 1,100, 900 and 750 bp), not being found in the patterns A, B and C. Pattern E differed from others in showing an increased number of major bands.

Monitoring of fungal diseases is essential to improve our understanding of their epidemiology and to enable research and prevention efforts to be more effective. In order to improve such monitoring, it is important to follow rigorous epidemiological methods. Here we describe the application of the RAPD assay to the most frequently causative pathogen of superficial mycoses, *T. rubrum* using random primers. Our research suggested that both primers (1 and 6), for the first time employed in dermatophyte typing, could be used to subtype *T. rubrum*. Genetic research has demonstrated that dermatophytes constitute an extremely homogeneous group. Some of the dermatophytes most frequently isolated have been identified by nested-PCR, using the genomic sequences of the DNA topoisomerase II genes[7]. However, there is a lack of easily methods available that reveal intraspecific polymorphisms suitable for epidemiological study of this fungus, characterization of molecular subtypes and therapeutic evaluation. The RAPD method of DNA fingerprinting has become quite popular for many infectious fungi and has been applied successfully[8]. However, the main problem is the reproducibility not only among laboratories, but also within a laboratory over times. In this study,

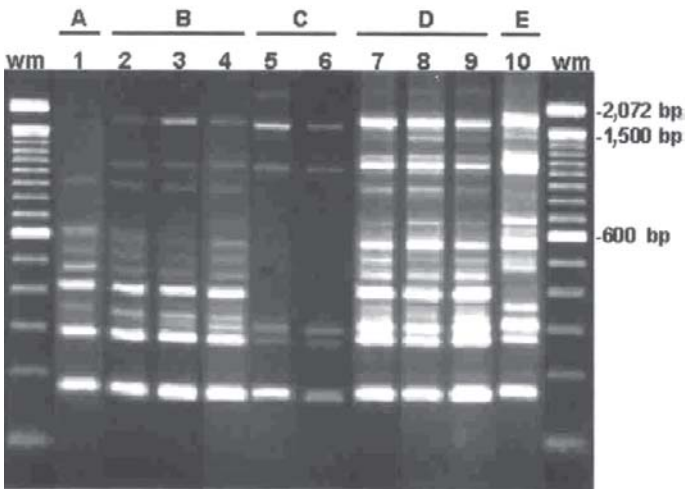

Fig. 1 - RAPD profiles of 10 *T. rubrum* strains obtained with primer 1. Electrophoresis of the RAPD products was done in a 2% agarose gel. Lanes: **wm,** molecular weight marker (Gibco 100 bp), size range, 2,072 to 100 bp; **1,** strain 2392; **2,** 2418; **3,** 2419; **4,** 2423; **5,** 2424; **6,** 2422; **7,** 2391; **8,** 2393; **9,** 2412; **10,** 2395. Similar profiles are indicated (A to E).

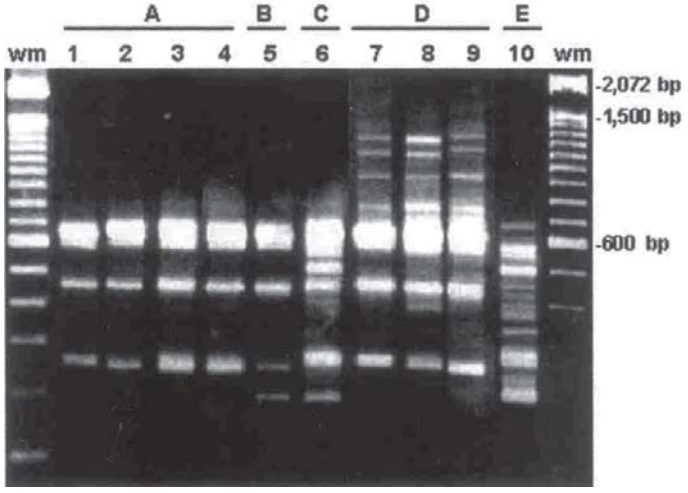

Fig. 2 - RAPD profiles of 10 *T. rubrum* strains obtained with primer 6. Electrophoresis of the RAPD products was done in a 2% agarose gel. Lanes: **wm,** molecular weight marker (Gibco 100 bp), size range, 2,072 to 100 bp; **1,** strain 2395; **2,** 2391; **3,** 2392; **4,** 2393; **5,** 2424; **6,** 2412; **7,** 2419; **8,** 2422; **9,** 2423; **10,** 2418. Similar profiles are indicated (A to E).

each sample of optimized genomic DNA was amplified in duplicate in repeated PCRs at different times; the DNA of two isolates were extracted from independent cultures and used in RAPD. The duplicate RAPD profiles showed identical band patterns (data not shown) and the reproducibility was 100%. The intraspecific polymorphisms achieved in our study differed from what is demonstrated in most of the available literature using RAPD. ZHONG *et al.*[15] examined thirty isolates of *T. rubrum* by RAPD and found 22 strains to be indistinguishable and eight to show very minor differences, while LIU *et al*.[8] using AP-PCR, reported no differences between eight strains of *T. rubrum*. The sequences applied in our study, probably hybridize with less conserved regions of the genomic DNA and generate a larger number of polymorphisms. Molecular methods, such as the analysis of restriction fragment length polymorphisms (RFLP)[3] and comparisons of sequences of the internal transcribed spacer 1 (ITS1) region ribosomal[9], are able to detect DNA differences between species and strains of dermatophytes, including *T. rubrum*. However, most of these techniques are complex, laborious, time-consuming, and not easily employable for routine work; in contrast, RAPD technology is simple, rapid and, in the absence of specific nucleotide sequence information for the many dermatophyte species, able to generate species-specific or intraspecific DNA polymorphisms on the basis of characteristic band patterns. Thus, we performed the intraspecific differentiation of *T. rubrum* in order to assist therapeutic and epidemiological studies of dermatomycosis. These variations in the genotype of *T. rubrum* have important implications for study design when looking at the epidemiology of dermatophyte infections.

RESUMO

Diferenciação de cepas de *Trichophyton rubrum* por amplificação randômica de DNA polimórfico (RAPD)

Trichophyton rubrum é um importante agente causal de dermatomicose. Os métodos de tipagem molecular têm sido recentemente desenvolvidos para responder questões sobre epidemiologia e auxiliar no esclarecimento de recidivas, após o tratamento. As seqüências aleatórias 1- (5'-d[GGTGCGGGAA]-3') e 6- (5'-d[CCCGTCAGCA]-3') foram usadas para tipagem molecular deste fungo por RAPD produzindo variabilidade intraespecífica. Cinco padrões foram observados entre os 10 isolados de *T. rubrum*, com ambas as seqüências. Foi concluído que a análise por RAPD pode ser utilizada para estudos epidemiológicos.

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