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

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15

16 **Abstract**

17

18 *Trichophyton rubrum* represents the most frequently isolated causative agent of superficial
19 dermatophyte infections. Several genotyping methods have recently been introduced to improve the
20 delineation between pathogenic fungi, both at the species and strain level. The purpose of this study
21 was to apply selected DNA fingerprinting methods to the identification and strain discrimination of *T.*
22 *rubrum* clinical isolates. Fifty-seven isolates from as many tinea patients were subjected to species
23 identification by PCR-restriction fragment length polymorphism (RFLP) analysis and strain
24 differentiation using a randomly amplified polymorphic DNA (RAPD) method, with two primers
25 designated 1 and 6. Using PCR-RFLP, 55 of the isolates studied were confirmed to be *T. rubrum*.
26 Among those, a total of 40 and 5 distinct profiles were obtained by RAPD with primer 1 and 6,
27 respectively. The combination of profiles from both RAPD assays resulted in 47 genotypes and an
28 overall genotypic diversity rate of 85.4%. A dendrogram analysis performed on the profiles generated
29 by RAPD with primer 1, showed most of the isolates (87.3%) to be genetically related. The PCR-
30 RFLP serves as a rapid and reliable method for the identification of *T. rubrum* species, while the
31 RAPD analysis is rather a disadvantageous tool for *T. rubrum* strain typing.

32

33 Key words: *Trichophyton rubrum*; dermatophytes; genotyping; PCR-RFLP; RAPD

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38 **Introduction**

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

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

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

56

57 **Material and Methods**

58

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

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

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

67 A rapid mini-preparation procedure was used to extract fungal genomic DNA [5]. One μL of purified
68 DNA (*ca* 20 ng) was used for each PCR assay.

69 Identification of *T. rubrum* was achieved by restriction fragment length polymorphism (RFLP)
70 analysis of the internal transcribed spacer (ITS) region of rDNA, as previously described [6]. The
71 amplified products were digested with *Mvn*I, *Hinf*I and where necessary with *Mva*I restriction
72 endonucleases (Roche), at a 2 hours incubation at 37°C. The resulting restriction fragments were

73 separated electrophoretically on 8% polyacrylamide gels and visualized under UV light after ethidium
74 bromide (Et-Br) staining.

75 Two primers, designated 1 and 6, as devised by Beaza and Mendes-Giannini [7] were used in two
76 separate RAPD assays. Amplification products were resolved by electrophoresis using 1.5% agarose
77 gels, and photographed under UV after Et-Br staining. The gel images were analysed by GelDoc
78 system and Quantity One (BioRad, USA) software. A dendrogram of the RAPD profiles obtained with
79 primer 1 was constructed using Dice's coefficient of similarity and the unweighted pair-group
80 arithmetic averaging (UPGMA) clustering method. An 80% genetic relatedness cutoff was used to
81 define clusters.

82

83 Results

84

85 Of the 57 clinical isolates assessed in this study and recognized as *T. rubrum* by conventional
86 identification methods, 55 (96.5%) were further confirmed as *T. rubrum* by means of PCR-RFLP
87 analysis. Using *MvnI*, all the isolates, except one (841/05), yielded restriction patterns consistent to
88 that of *Trichophyton* taxon. Using *HinfI*, 55 (96.5%) isolates produced fragments whose sizes
89 coincided with those expected for *T. rubrum*. One isolate (899/05) generated a pattern corresponding
90 to either *T. mentagrophytes* var. *interdigitale* or *T. tonsurans*. The isolate was eventually classified as
91 *T. tonsurans*, based upon *MvaI* restriction analysis. One isolate (841/05) could not be assigned to any
92 of the dermatophyte species, distinguishable by their unique RFLP profiles obtained with *MvnI*, *HinfI*,
93 or *MvaI* enzymes.

94 Among the 55 *T. rubrum* isolates, a total of 40 distinct patterns (A-AN) were obtained by RAPD with
95 primer 1. Two additional patterns were observed for non-*T. rubrum* isolates (designated as BB and CC
96 for isolates no. 841/05 and 899/05, respectively). The four most prevalent patterns, designated as I,
97 AJ, AE, and AN, contained 5, 4, 3, and 3 isolates respectively. Four patterns (C, AF, AG, and AI)
98 contained two isolates each, and the remaining 34 patterns were represented by single isolates. The
99 RAPD with primer 6 generated five different profiles in *T. rubrum* isolates (a-e), and two additional
100 profiles in non-*T. rubrum* isolates (designated as "f" and "g" for isolates no. 841/05 and 899/05,
101 respectively). Among the *T. rubrum* isolates, the most abundant RAPD profile was that designated as
102 "b", found in 23 isolates, followed by profiles designated as "a" and "c", represented by 19 and 11
103 isolates, respectively. Patterns designated as "d" and "e" were each identified for one isolate.

104 The combination of the profiles obtained with both primers resulted in 47 different genotypes for the
105 *T. rubrum* isolates. Six of those genotypes were common to 3 (type I-a and type AJ-a) or 2 (types: C-
106 a, AE-b, AF-b, AN-a) isolates, whereas the remaining 41 genotypes were unique, that is represented
107 by single isolates only.

108 The distribution of the genotypes varied among the three geographical regions, from which the isolates
109 originated. Of the 40 RAPD genotypes produced with primer 1, each was restricted to only one

110 geographical location. Twenty-six genotypes occurred in isolates from Lower Silesia, 8 genotypes
111 were observed in isolates from Cracow, and 6 – in isolates from Tübingen, Germany. Regarding the
112 genotypes obtained by RAPD with primer 6, genotypes “b” and “c” were found in isolates from all
113 three regions studied, and genotype “a” was observed in the Lower-Silesian and Tübingen isolates, but
114 not in the Kraków isolates. The genotypes “a”, “b”, and “c” were the most prevalent in single regions,
115 i. e. in Lower Silesia (44.7% of isolates), Kraków (62.5%), and Tübingen (44.4%), respectively.

116 A dendrogram based on the RAPD profiles with primer 1, allowed the separation of the *T. rubrum*
117 isolates into genetic similarity groups (clusters). A total of 48 isolates could be allocated into six main
118 clusters (I-VI) with the similarity index between the isolates within the cluster being 80% or higher
119 (Fig. 1). The largest cluster (VI) comprised 21 isolates, for which 11 different patterns were obtained.
120 Clusters IV and V consisted of 8 isolates each, being representative of either 4 (cluster IV) or 8
121 (cluster V) distinct patterns. The numbers of isolates (and corresponding patterns) belonging to the
122 remaining clusters were 3 (2 patterns) for cluster I, 5 (5) for cluster II, and 3 (3) for cluster III.

123

124 Discussion

125

126 The traditional, culture-based methods of identifying dermatophytes are cumbersome, laborious, and
127 often inconclusive, due to fungal phenotypic variability and pleomorphism [1, 8]. However, the advent
128 of molecular biology tools has enabled the development of new molecular approaches to the diagnosis
129 of dermatophyte infections. One such approach, introduced by Jackson et al., relies upon RFLP
130 analysis of PCR amplified ITS regions of the rDNA gene complex [9]. This PCR-RFLP strategy was
131 also employed in the present study. The only modification to the original procedure was the use of
132 *MvnI* and *HinfI* instead of *MvaI*. Whereas digestion with *MvnI* allows discrimination between the three
133 main dermatophyte genera (*Trichophyton*, *Microsporum*, and *Epidermophyton*) [10], the *HinfI*
134 digestion results in species-specific restriction profiles [11]. By using those two restriction enzymes,
135 we wanted to verify their usefulness in the molecular identification of dermatophyte species, and *T.*
136 *rubrum* in particular. The results from this study showed high concordance between conventional and
137 molecular techniques for identifying *T. rubrum*. Only two isolates were identified as non-*T. rubrum*
138 isolates: one was identified as *T. tonsurans* (upon restriction analysis with *MvaI*), and the other was
139 concluded to be a non-dermatophyte fungus. A possible explanation for the latter may relate to the
140 laboratory contamination of the specimen and/or culture. It is also to note that the application of the
141 PCR-RFLP analysis, as in this study, does not allow to distinguish between *T. rubrum* and *T.*
142 *soudanense* [9]. The distinction between the two species is only possible with methods that target
143 single nucleotide polymorphisms (SNPs) within the ITS regions of rDNA [12]. Given however that *T.*
144 *rubrum* differs substantially from *T. soudanense* in terms of phenotypic properties and geographical
145 distribution (the former is cosmopolitan, while the latter is restricted mainly to the sub-Saharan part of
146 Africa), the assignment to the *T. rubrum* species, provided by PCR-RFLP seems unambiguous.

147 Differentiation of *T. rubrum* at the strain level has been attempted using a number of genotyping
148 methods, though with unsatisfactory results [3, 13, 14]. However, Jackson et al., have recently
149 reported on intraspecific variability within *T. rubrum* by PCR amplifying two tandemly repetitive
150 subelements (TRSs), located in the non-transcribed spacer (NTS) region of the rRNA gene cluster
151 [15]. More recently, the RAPD analysis, performed by Baeza et al., with two decameric primers,
152 designated 1 and 6, has been shown to produce a high degree of inter-strain polymorphism [7, 16].
153 The RAPD analysis with primers 1 and 6 was also applied in the present study, and this choice was
154 motivated by a high discriminatory potential of the method, higher than that of the TRS typing system
155 [16]. The RAPD typing demonstrated a high genetic diversity of the *T. rubrum* population studied.
156 Based on the combined RAPD profiles, a total of 47 distinct genotypes were obtained. Hence, the
157 overall genetic diversity rate (GDR), calculated as the number of different genotypes divided by the
158 number of isolates, was 85.4%. It is noteworthy that most of the polymorphism was generated by
159 RAPD with primer 1. It yielded 40 profiles, whereas RAPD with primer 6 resulted in only 5 profiles
160 (GDRs of 72.7% and 9.1%, respectively). In the first study that used primers 1 and 6, among 10
161 clinical isolates of *T. rubrum*, 5 molecular patterns were observed for each primer [7]. In a subsequent
162 study including 67 *T. rubrum* isolates, a total of 12 and 11 individual patterns were obtained by RAPD
163 with primer 1 and 6, respectively (GDRs of 17.9% and 16.4%, respectively) [16]. Another study that
164 investigated the intraspecific diversity of *T. rubrum* isolates originating from Japan and China revealed
165 a considerable tightness of the population structure. All 150 isolates tested were split into only 19
166 fingerprinting genotypes, based on the combined RAPD analyses with primer 1 and 6 (GDR of 12.7%)
167 [17]. Much higher genotypic variability with the same primers was shown in a recent study of Santos
168 et al. [18]. Nineteen different molecular profiles were configured for 52 *T. rubrum* isolates, when each
169 of the primers was used independently, resulting in a GDR of 36.5%. It was speculated by the authors
170 that the greater genetic diversity revealed in their study might result from having used strains
171 exclusively from patients with onychomycosis. This condition, with its frequent chronicity, has been
172 associated with mixed infections by multiple *T. rubrum* genotypes [19]. Collectively, the results of the
173 studies cited above differ in terms of genetic polymorphism achieved by RAPD with both of the
174 primers. The polymorphism obtained in our study was exceptionally high, and this may relate to the
175 specific, genetic structure of Polish (and German) isolates, different from that of the so far analysed *T.*
176 *rubrum* populations.

177 The polymorphism obtained by RAPD with primer 1 was further investigated by a dendrogram
178 analysis derived from the similarity coefficients between the RAPD patterns. Although the similarity
179 coefficient value for the entire *T. rubrum* population studied was calculated at 52%, a vast majority of
180 isolates (87.3%) were distributed into six clusters, within which all the isolates shared at least 80%
181 similarity. This finding, considering that the similarity coefficient value of 0.8-0.99 is assumed to
182 represent genetically related isolates [20], indicates that the clustered isolates might have originated
183 from the same strain that had undergone microevolutionary changes [16]. Thus, the high

184 polymorphism resolved by RAPD may relate to the reproducibility of the RAPD technique itself.
185 Indeed, RAPD assays often suffer from poor reproducibility and variations in the amplification
186 patterns between isolates may be caused by even the slightest changes in the PCR reaction conditions.
187 This explains the reluctance on the part of researchers to use the RAPD method and their search for
188 newer molecular tools with a better diagnostic performance. One such promising alternative method is
189 multilocus microsatellite typing (MLMT) which has recently been developed by Gräser et al. [4].

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

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

201

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207

208 **References**

209

- 210 1. Weitzman I, Summerbell RC (1995) The dermatophytes. Clin Microbiol Rev 8:240-259.
- 211 2. Tietz H-J, Kunzelmann V, Schönian G (1995) Changes in fungal spectrum of
212 dermatomycoses. Mycoses 38:33-39.
- 213 3. Gräser Y, Kühnisch J, Presber W (1999) Molecular markers reveal exclusively clonal
214 reproduction in *Trichophyton rubrum*. J Clin Microbiol 37:3713-3717.
- 215 4. Gräser Y, Fröhlich J, Presber W, de Hoog S (2007) Microsatellite markers reveal geographic
216 population differentiation in *Trichophyton rubrum*. J Med Microbiol 56:1058-1065.
- 217 5. Liu D, Coloe S, Baird R, Pedersen J (2000) Rapid mini-preparation of fungal DNA for PCR. J
218 Clin Microbiol 38:471.

- 219 6. White TJ, Burns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal
220 ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ
221 (Eds.) PCR protocols: a guide to methods and applications. San Diego, CA: Academic Press
222 Inc. pp 315-322.
- 223 7. Baeza LC, Mendes-Giannini MJS (2004) Strain differentiation of *Trichophyton rubrum* by
224 random amplification of polymorphic DNA (RAPD). Rev Inst Med Trop S Paulo 46:339-341.
- 225 8. Gräser Y, Kuijpers AF, Presber W, De Hoog GS (2000) Molecular taxonomy of the
226 *Trichophyton rubrum* complex. J Clin Microbiol 38:3329–3336.
- 227 9. Jackson CJ, Barton RC, Evans EG (1999) Species identification and strain differentiation of
228 dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J Clin Microbiol
229 37:931-936.
- 230 10. Dobrowolska A, Stączek P, Kaszuba A, Kozłowska M (2006) PCR-RFLP analysis of the
231 dermatophyte isolates from patients in Central Poland. J Dermatol Sci 42:71-74.
- 232 11. Mochizuki T, Tanabe H, Kawasaki M, Ishizaki H, Jackson CJ (2003) Rapid identification of
233 *Trichophyton tonsurans* by PCR-RFLP analysis of ribosomal DNA regions. J Dermatol Sci
234 32:25-32.
- 235 12. Kong F, Tong Z, Chen X et al (2008) Rapid identification and differentiation of *Trichophyton*
236 species, based on sequence polymorphisms of the ribosomal internal transcribed spacer
237 regions, by rolling-circle amplification. J Clin Microbiol 46:1192-1199.
- 238 13. Liu D, Coloe S, Pedersen J, Baird R (1996) Use of arbitrarily primed polymerase chain
239 reaction to differentiate *Trichophyton* dermatophytes. FEMS Microbiol Lett 136:147-150.
- 240 14. Zhong Z, Li R, Li D, Wang D (1997) Typing of common dermatophytes by random
241 amplification of polymorphic DNA. Jpn J Med Mycol 38:239-246.
- 242 15. Jackson CJ, Barton RC, Kelly SL, Evans EGV (2000) Strain identification of *Trichophyton*
243 *rubrum* by specific amplification of subrepeat elements in the ribosomal DNA nontranscribed
244 spacer. J Clin Microbiol 38:4527-4534.
- 245 16. Baeza LC, Matsumoto MT, Almeida AM, Mendes-Giannini MJS (2006) Strain differentiation
246 of *Trichophyton rubrum* by randomly amplified polymorphic DNA and analysis of rDNA
247 nontranscribed spacer. J Med Microbiol 55:429-436.
- 248 17. Yang X, Sugita T, Takashima M, Hiruma M, Li R, Sudo H, Ogawa H, Ikeda S (2009)
249 Differentiation of *Trichophyton rubrum* clinical isolates from Japanese and Chinese patients
250 by randomly amplified polymorphic DNA and DNA sequence analysis of the non-transcribed
251 spacer region of the rRNA gene. J Dermatol Sci 54:38-42.
- 252 18. Santos DA, Araújo RA, Hamdan JS, Cisalpino PS (2010) *Trichophyton rubrum* and
253 *Trichophyton interdigitale*: genetic diversity among species and strains by random amplified
254 polymorphic DNA method. Mycopathologia 169:247-255.

- 255 19. Santos DA, Araújo RA, Kohler LM, Machado-Pinto J, Hamdan JS, Cisalpino PS (2007)
256 Molecular typing and antifungal susceptibility of *Trichophyton rubrum* isolates from patients
257 with onychomycosis pre- and post-treatment. *Int J Antimicrob Agents* 29:563-569.
- 258 20. Marol S, Yücesoy M (2008) Molecular epidemiology of *Candida* species isolated from
259 clinical specimens of intensive care unit patients. *Mycoses* 51:40-49.
- 260 21. Ohst T, de Hoog S, Presber W, Stavrakieva V, Gräser Y (2004) Origins of
261 microsatellite diversity in the *Trichophyton rubrum*-*T. violaceum* clade
262 (dermatophytes). *J Clin Microbiol* 42:4444-4448.

263

264 **Figure legend**

265

266 **Fig. 1** Dendrogram showing genetic relationships among 55 *T. rubrum* strains inferred from the RAPD
267 patterns, generated by using primer 1

268

