Journal of Medical Microbiology (2010), **59**, 185–192 DOI 10.1099/jmm.0.013458-0

Evaluation of a PCR melting profile method for intraspecies differentiation of *Trichophyton rubrum* and *Trichophyton interdigitale*

Justyna Leibner-Ciszak,[1]† Anita Dobrowolska,[2]† Beata Krawczyk,[1] Aleksandra Kaszuba[3] and Paweł Stączek[2]

Correspondence
Justyna Leibner-Ciszak
justyna.leibner@wp.pl

[1]Gdansk University of Technology, Chemical Faculty, Department of Microbiology, ul. Narutowicza 11/12, 80-233 Gdansk, Poland

[2]University of Lodz, Department of Genetics of Microorganisms, ul. Banacha 12/16, 90-237 Lodz, Poland

[3]Medical University of Lodz, Department of Dermatology and Venerology, ul. Kniaziewicza 1/5, 91-347 Lodz, Poland

In order to identify the source of infections caused by dermatophytes, as well as the pathogen transmission pathway, there is a need to determine methods that allow detailed genetic differentiation of the strains within the dermatophyte genera. In this work, a PCR melting profile (PCR-MP) technique based on the ligation of adaptors and the difference in melting temperatures of DNA restriction fragments was used for the first time for intraspecies genotyping of dermatophytes. Clinical isolates and reference strains of dermatophytes isolated from skin, scalp, toenails and fingernails were used for this study. PCR-MP and random amplification of polymorphic DNA (RAPD) were used to type 11 isolates of *Trichophyton rubrum*, 40 isolates of *Trichophyton interdigitale* and 14 isolates of *Microsporum canis*. The results distinguished five types (containing one subtype) characteristic for *T. rubrum* and seven types characteristic for *T. interdigitale* using the PCR-MP technique. Analysis conducted using RAPD revealed five types for *T. rubrum* and four types for *T. interdigitale* isolates. No differentiation was observed for the *M. canis* isolates with either method. These results demonstrate that PCR-MP is a reliable method for the differentiation of *T. rubrum* and *T. interdigitale* strains and yields a discriminatory power that is at least equal to that of RAPD.

Received 8 June 2009
Accepted 3 November 2009

INTRODUCTION

Dermatophytes, a group of pathogenic fungi, have a high affinity for keratinized structures such as skin, hair and nails, and are responsible for the development of the diseases known as dermatophytoses (Weitzman & Summerbell, 1995).

In order to identify the source of infections, as well as pathogen transmission pathways and whether the original isolate is responsible for re-infection, there is a need to establish methods that allow detailed genetic differentiation of the strains within the dermatophyte genera. Moreover, differentiation of dermatophytes at the strain level can help to identify whether multiple lesions are caused by the same or different strains, can be important in treatment and prophylaxis, and can aid in the exploration of population structure and the distribution of strains in various geographical regions (Abdel-Rahman, 2008). Several genotyping techniques such as PCR, random amplification of polymorphic DNA (RAPD), random amplification of monomorphic DNA (Baeza & Giannini, 2004; Baeza *et al.*, 2006; Kac *et al.*, 1999; Kaszubiak *et al.*, 2004) and PCR-RFLP of the non-transcribed spacer rRNA gene region or tandemly repetitive subelements from this region (TRS-1) have been applied to analyse *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans* and *Microsporum canis* (de Assis Santos *et al.*, 2007; Gräser *et al.*, 2000; Kamiya *et al.*, 2004; Mochizuki *et al.*, 2003; Yazdanparast *et al.*, 2003).

These methods have a relatively high discriminatory power and good reproducibility, but they are still not suited to applications designed to establish associations between strain types and their geographical distribution (Jackson *et al.*, 2000), and they cannot help to describe the real nature of dermatophyte infections, especially whether recurrent infections are due to involvement of a new strain or reactivation of an old one (Jackson *et al.*, 2000).

†These authors contributed equally to this paper.

Abbreviations: MP, melting profile; RAPD, random amplification of polymorphic DNA.

In this study, we utilized a relatively new PCR melting profile (PCR-MP) technique based on the method of ligation-mediated PCR. This technique was developed by Masny & Płucienniczak (2003) and Krawczyk *et al.* (2006) for bacterial strain differentiation. The discriminatory factor of the method is a lowering of the denaturation temperature during PCR, when only less stable DNA fragments (lower G+C content) are amplified. An outline of the procedure is depicted in Fig. 1. The PCR-MP method was implemented using a set of clinical *T. rubrum*, *T. interdigitale* and *M. canis* strains isolated from patients in Poland and Denmark. The utility of the PCR-MP method was estimated by comparison with data obtained using RAPD.

METHODS

Fungal strains. Eleven isolates of *T. rubrum*, 40 of *Trichophyton interdigitale* and 14 of *M. canis* were used in this study (Table 1). They were isolated from skin, scalp, toenails and fingernails. Standard mycological identification according to the features of fungal colonies and their morphology observed under a microscope was performed at the Department of Dermatology and Venerology, Medical University of Lodz, and in the Department of Bacteriology, Mycology and Parasitology of the Statens Serum Institute in Denmark. Traditional identification was confirmed by PCR-RFLP identification targeting the ITS1–5.8S–ITS2/*Hin*fI region (Dobrowolska *et al.*, 2006; Mochizuki *et al.*, 2003) and by sequencing of randomly selected PCR products (ITS1–5.8S–ITS2) of *T. rubrum* strain numbers 1 and 3, *T. interdigitale* strain numbers 1, 5, 10, 20, 30 and 40, and *M. canis* strain numbers 1 and 10 (Table 1). The results obtained were compared with GenBank data. Reference strains used in this study originated from the Centraalbureau voor Schimmelcultures (CBS) collection.

DNA extraction. Total cellular DNA was extracted from a small amount of mycelium cultured on Sabouraud agar slants, by a slightly self-modified rapid mini-preparation method (Liu *et al.*, 2000). Mycelium was suspended in 700 µl lysis buffer [400 mM Tris/HCl (pH 8.0), 60 mM EDTA, 150 mM NaCl, 1 % SDS] and incubated at 60 °C for 1 h. After the addition of 210 µl 3 M sodium acetate, the homogenate was centrifuged at 7500 ***g*** for 15 min. The supernatant was successively extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The DNA was treated with RNase at a final concentration of 50 µg ml^{-1} for 20 min at 57 °C. The samples were then precipitated using 3 vols cold ethanol in the presence of 300 mM sodium acetate and the DNA was centrifuged for 10 min. The pellet was washed with 70 % ethanol and air dried. The DNA was dissolved in 50 µl Tris/EDTA buffer, and 1 µl of the resulting solution was used as template in the following PCR.

PCR-MP. The PCR-MP procedure, which was initially developed for *Escherichia coli* differentiation (Krawczyk *et al.*, 2006), was optimized for dermatophyte differentiation, using varying amounts of reagents as well as time variations of the particular stages. In this study, we used four restriction enzymes (and their recommended buffers) in the digestion step: *Hin*dIII (Buffer R; Fermentas), *Bam*HI (Buffer B; Roche Applied Science), *Eco*RI (Buffer A; Roche Applied Science) and *Sal*I (Buffer O; Fermentas). For digestion of genomic DNA, approximately 40–300 ng of the DNA sample was added to the reaction tube containing 2.5 µl restriction buffer and 0.4 µl (4 U) of the appropriate endonuclease in a total volume of 25 µl. After incubation at 37 °C for 15 min, the following ligation mix was added: 15 pmol of two oligonucleotides (helper + ligated) to create an appropriate adaptor (Table 2), 2.5 µl ligation buffer [400 mM Tris/HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM dithiothreitol, 5 mM ATP; Fermentas] and 0.5 µl T4 DNA ligase (0.5 U; Fermentas). The samples were then incubated at 22 °C for 15 min. PCR was carried out in a 25 µl reaction mixture containing 1 µl ligation solution, 2.5 µl 10 × PCR buffer Shark [200 mM Tris/HCl (pH 8.8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1 % Triton X-100; DNA Gdańsk], 2.25 µl dNTP mix (2 mM each dNTP), 0.5 µl (1 U) *Pwo* polymerase *Hypernova* (DNA Gdańsk) and 25 pmol appropriate primer (Table 2). The PCR primers were longer (extended by a protruding 5′-end sequence and the remaining part of the restriction site) than the ligated oligonucleotides in order to overcome the PCR suppression phenomenon.

The denaturation temperature was determined by specific optimization experiments with reference and a number of clinical *T. rubrum* isolates, using a gradient thermal cycler (Mastercycler ep gradient S; Eppendorf) within a gradient range of 78.0–83.0 °C for *Hin*dIII restriction system and 84.0–86.2 °C for *Bam*HI restriction system. The PCRs were performed as follows: 7 min at 72 °C; initial denaturation for 90 s over a gradient of 78.0–83.0 °C; 24 cycles of denaturation for 1 min at a gradient of 78.0–83.0 °C, annealing and elongation step at 72 °C for 2 min 15 s; and a final elongation at 72 °C for 5 min.

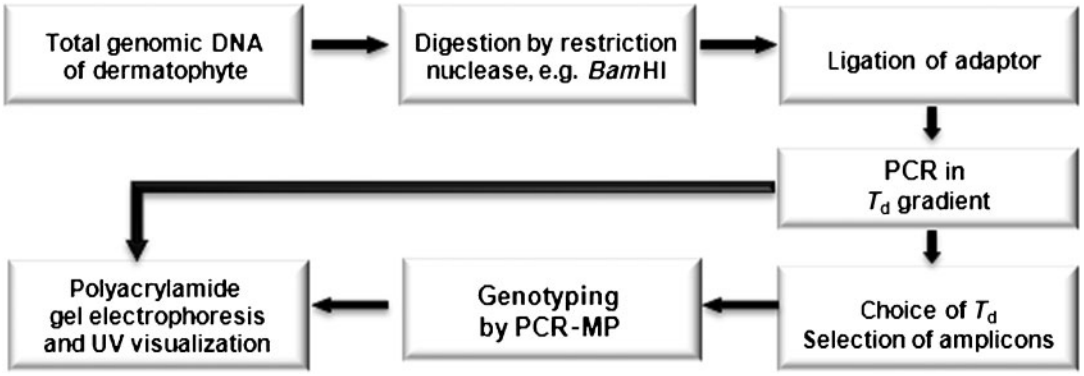

Fig. 1. Diagram illustrating the PCR-MP technique used in this study. T_d, Denaturation temperature.

For all isolates of *T. rubrum*, the PCRs were performed as described above, using the established optimal denaturation temperatures of 78.6 and 84.3 °C, respectively, for *Hin*dIII and *Bam*HI.

The denaturation temperatures for *T. interdigitale* isolates were calculated during the optimization experiments over a gradient range of 75.0–78.0 °C for *Hin*dIII and 84.1–88.0 °C for *Bam*HI. Analysis of all isolates was performed using the established optimal denaturation temperatures of 77.7 and 84.4 °C, respectively, for *Hin*dIII and *Bam*HI.

Isolates of *M. canis* were examined by the PCR-MP method using four restriction enzymes at the following gradient ranges: *Hin*dIII, 81.0–85.0 °C; *Bam*HI, 83.8–87.7 °C; *Eco*RI, 80.5–85.5 °C; and *Sal*I, 84.0–88.0 °C. All isolates were then analysed at the established optimal denaturation temperature of 81.3, 85.7, 82.9 or 86.7 °C, respectively.

All analyses were performed at least in triplicate, and PCRs were performed using three different thermal cyclers: a Mastercycler ep gradient S (Eppendorf), a TGradient thermal cycler (Biometra) and a Perkin-Elmer Applied Biosystem GeneAmp PDR System 2400.

Electrophoresis of all PCR products was performed on 6 % polyacrylamide gels.

RAPD. Isolates of *T. rubrum* were typed by RAPD using Primer-1 (Baeza *et al.*, 2006; Table 2). Amplifications were performed with approximately 100 ng *T. rubrum* DNA in 25 µl reaction mixture containing 2.5 µl 10× PCR buffer with KCl (Fermentas), 4 µl 25 mM $MgCl_2$, 0.75 U *Taq* DNA polymerase (Fermentas), 2.5 µl dNTP mixture (2.5 mM each) and 0.25 µl primer (100 µM). PCRs were performed in a TGradient thermal cycler as follows: initial denaturation at 94 °C for 2 min; five cycles of denaturation at 94 °C, annealing at 25 °C for 5 min and extension at 72 °C for 2 min; and 30 cycles of denaturation at 94 °C for 1 min, annealing at 30 °C for 1 min and extension at 72 °C for 2 min. After the last cycle, a final extension step was performed at 72 °C for 2 min.

The isolates of *T. interdigitale* were also analysed by RAPD using primer A08 (Kac *et al.*, 1999; Table 2). PCR was carried out in a TGradient thermal cycler in 25 µl reaction volumes, each containing 10–100 ng genomic DNA, 2 µM A08 primer, 2.5 µl 10× PCR buffer with KCl, 2 µl 25 mM $MgCl_2$, 0.75 U *Taq* DNA polymerase (Fermentas) and 2.5 µl dNTP mix (2.5 mM each). The reaction was performed as described previously (Kac *et al.*, 1999).

Typing of *M. canis* isolates by RAPD was conducted with primers OPI07 and OPK20 (Yu *et al.*, 2004; Table 2). The reaction mixtures contained 2.5 µl 10× PCR buffer with KCl, 2 µl 25 mM $MgCl_2$, 0.75 U *Taq* DNA polymerase (Fermentas), 2.5 µl dNTP mix (2.5 mM each) and 20 pmol each primer (100 µM). PCR was performed as described previously (Yu *et al.*, 2004).

All *M. canis* isolates were also examined by RAPD as described above for the *T. rubrum* and *T. interdigitale* isolates.

All amplification products were analysed by electrophoresis of 10 µl samples on 6 % polyacrylamide gels.

DNA analysis. Strains with identical sizes and numbers of well-defined bands in the gel were considered to be genetically indistinguishable and were assigned to the same type. Strains with banding patterns that differed by up to three bands were considered to be closely related and were described as subtypes. Strains with banding patterns that differed by four or more bands were considered to be different types. The patterns obtained from the electropherograms by the three methods used were also converted and analysed using Quantity One software, version 4.3.1 (Bio-Rad).

RESULTS

T. rubrum and *T. interdigitale* typing

All isolates of *T. rubrum* and *T. interdigitale* were analysed with two restriction enzymes, *Hin*dIII and *Bam*HI. Both gave compatible PCR-MP fingerprints for the examined isolates. The PCR-MP fingerprinting patterns obtained for the *Bam*HI restriction system are presented in Fig. 2(a).

Among the 11 isolates of *T. rubrum*, we distinguished five types (A–E) by PCR-MP and RAPD analysis (Table 1). Genotype A was markedly predominant and was characteristic of seven isolates of *T. rubrum* originating from Poland. The PCR-MP technique was able to differentiate one subtype (A1) among genotype A. The remaining isolates were classified as four different genotypes (B–E), each represented by one isolate.

In the case of the *T. interdigitale* isolates, PCR-MP analysis revealed seven genotypes (A–G) for both restriction systems (Table 1). Representative PCR-MP patterns for the *Hin*dIII restriction system are shown in Fig. 3(a). The RAPD technique displayed a lower discrimination power than that of PCR-MP. Among all of the *T. interdigitale* isolates, we distinguished only four genotypes using RAPD (Table 1).

All 29 isolates of *T. interdigitale* originating from Poland (Lodz) were classified into three genetic groups (A, C and D) by PCR-MP and RAPD. Genotype A was predominant and contained 21 of the analysed isolates. Genotype C (with one subtype C1 distinguished for PCR-MP analysis) was characteristic for three isolates, and five isolates of *T. interdigitale* were assigned to genotype D (Table 1).

For the ten isolates of *T. interdigitale* originating from Denmark (Copenhagen), we were able to distinguish three characteristic patterns (E, F and G) by PCR-MP analysis, which were different from the genotypes determined for the isolates from Poland (Table 1). Genotype E (containing one subtype E1) was predominant and contained seven isolates of *T. interdigitale*, genotype F was specific for only one isolate, and two isolates belonged to genotype G. RAPD turned out to be less discriminative, as it revealed only two genotypes (A and C), identical to the genotypes identified in Poland. Genotype B was represented only by the reference strain of *T. interdigitale*.

Comparative pair-wise analysis of the PCR-MP patterns obtained for the *T. rubrum* and *T. interdigitale* isolates was performed using the Dice band-based similarity coefficient (Figs 2b and 3b). For isolates that were classified as unrelated, the Dice coefficient was <0.82.

M. canis typing

All 13 isolates of *M. canis* originating from Lodz and the reference strain were analysed by PCR-MP using four restriction enzymes (*Hin*dIII, *Bam*HI, *Eco*RI and *Sal*I). In all cases, we were able to distinguish only one genotype.

Table 1. Typing results from PCR-MP and RAPD analysis of genomic DNA for *T. rubrum*, *T. interdigitale* and *M. canis* strains

Strain name/no.	Morphological and molecular identification	Source of strain	Geographical localization	PCR-MP genotype	RAPD genotype
T. rubrum					
Anthropophilic strains					
1	*T. rubrum*	Tinea glabrae	Lodz, Poland	A	A
2	*T. rubrum*	Tinea unguium	Lodz, Poland	B	B
3	*T. rubrum*	Onychomycosis	Lodz, Poland	C	C
4	*T. rubrum*	Onychomycosis	Lodz, Poland	A1	A
5	*T. rubrum*	Onychomycosis	Lodz, Poland	A1	A
6	*T. rubrum*	Onychomycosis	Lodz, Poland	A1	A
7	*T. rubrum*	Tinea glabrae	Lodz, Poland	A1	A
8	*T. rubrum*	Onychomycosis	Wrocław, Poland	D	D
9	*T. rubrum*	Onychomycosis	Wrocław, Poland	A1	A
10	*T. rubrum*	Onychomycosis	Wrocław, Poland	A1	A
11	*T. rubrum* CBS 120358	Tinea unguium	Lausanne, Switzerland	E	E
T. interdigitale					
Anthropophilic strains					
1	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
2	*T. interdigitale*	Onychomycosis	Lodz, Poland	A	A
3	*T. interdigitale*	Onychomycosis	Lodz, Poland	A	A
4	*T. interdigitale*	Onychomycosis	Lodz, Poland	A	A
5	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
6	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
7	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
8	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
9	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
10	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
11	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
12	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
13	*T. interdigitale*	Tinea unguium	Lodz, Poland	A	A
14	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
15	*T. interdigitale* CBS 120357	Tinea capitis	Lausanne, Switzerland	B	B
22	*T. interdigitale*	Tinea glabrae	Lodz, Poland	C	C
23	*T. interdigitale*	Onychomycosis	Lodz, Poland	C1	C
24	*T. interdigitale*	Onychomycosis	Lodz, Poland	C	C
26	*T. interdigitale*	Tinea unguium	Lodz, Poland	D	D
27	*T. interdigitale*	Tinea unguium	Lodz, Poland	D	D
28	*T. interdigitale*	Tinea glabrae	Lodz, Poland	D	D
29	*T. interdigitale*	Tinea unguium	Lodz, Poland	D	D
30	*T. interdigitale*	Tinea unguium	Lodz, Poland	D	D
Zoophilic strains					
16	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
17	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
18	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
19	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
20	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
21	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
25	*T. interdigitale*	Tinea glabrae	Lodz, Poland	A	A
31	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	E	A
32	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	E	A
33	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	E	A
34	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	E1	C
35	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	E	A
36	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	G	A
37	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	E	A
38	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	E	A
39	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	F	A
40	*T. interdigitale*	Tinea capitis	Copenhagen, Denmark	G	A

Table 1. cont.

Strain name/no.	Morphological and molecular identification	Source of strain	Geographical localization	PCR-MP genotype	RAPD genotype
M. canis					
Zoophilic strains					
1	*M. canis*	Tinea unguium	Lodz, Poland	A	A
2	*M. canis*	Tinea unguium	Lodz, Poland	A	A
3	*M. canis*	Tinea unguium	Lodz, Poland	A	A
4	*M. canis*	Tinea unguium	Lodz, Poland	A	A
5	*M. canis*	Tinea unguium	Lodz, Poland	A	A
6	*M. canis*	Onychomycosis	Lodz, Poland	A	A
7	*M. canis*	Onychomycosis	Lodz, Poland	A	A
8	*M. canis*	Tinea unguium	Lodz, Poland	A	A
9	*M. canis*	Onychomycosis	Lodz, Poland	A	A
10	*M. canis*	Tinea unguium	Lodz, Poland	A	A
11	*M. canis*	Tinea glabrae	Lodz, Poland	A	A
12	*M. canis*	Tinea unguium	Lodz, Poland	A	A
13	*M. canis*	Tinea glabrae	Lodz, Poland	A	A
14	*M. canis* CBS 113480	Tinea capitis	Wurzburg, Germany	A	A

The same results were obtained by RAPD with three random primers. Additionally, to confirm these results, we compared the PCR-MP denaturation temperature profiles of the reference strain and one of the clinical isolates of *M. canis* for all restriction systems used. Identical profiles were obtained for both isolates. Representative results obtained for *Bam*HI are shown in Fig. 4.

The reproducibility of PCR-MP used for *T. rubrum*, *T. interdigitale* and *M. canis* was examined by performing four separate PCR-MP fingerprinting runs using three different thermal cyclers. In all cases, identical patterns were obtained.

DISCUSSION

New typing methods for dermatophytes can provide crucial insights into their epidemiology, population biology and pathogenicity. One of the main applications is identifying endemic strains that are characteristic for a certain area. Typing methods can identify the source of

Table 2. Oligonucleotides and PCR primers used in this study (complementary sequences are underlined)

Oligonucleotide/primer	Sequence (5′→3′)
PCR-MP	
Helper oligonucleotide	
*Hin*dIII	AGCTGTCGACGTTGG
*Bam*HI	GATCGTCGACGTTGG
*Eco*RI	AATTGTCGACGTTGG
*Sal*I	TCGAGTCGACGTTGG
Ligated oligonucleotide	
PowieTm	CTCACTCTCACCAACAACGTCGAC
MP primer	
PowaAGCTT	CTCACTCTCACCAACGTCGACAGCTT
MP *Bam*HI	CTCACTCTCACCAACGTCGACGATCC
MP *Eco*RI	CTCACTCTCACCAACGTCGACAATTC
MP *Sal*I	CTCACTCTCACCAACGTCGACTCGAC
RAPD	
Primer-1	GGTGCGGGAA
A08	GTGACGTAGC
OPI07	CAGCGACAAG
OPK20	GTGTCGCGAG

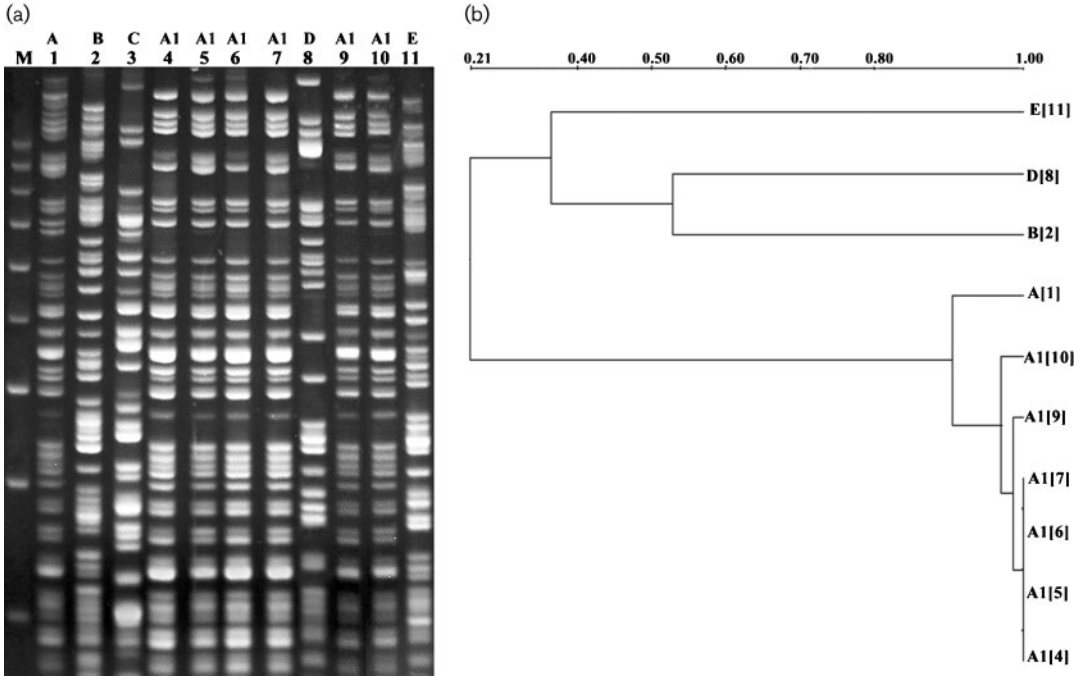

Fig. 2. PCR-MP fingerprints (a) and unweighted pair group method with arithmetic mean (UPGMA) dendrogram (b) obtained for *T. rubrum* isolates using *Bam*HI. Lane M, molecular size markers (1000, 900, 800, 700, 600, 500, 400, 300 and 200 bp). The number of isolates and PCR-MP types (assigned as in Table 1) are given above each lane. Electrophoresis of the DNA amplicons was carried out on a 6 % polyacrylamide gel.

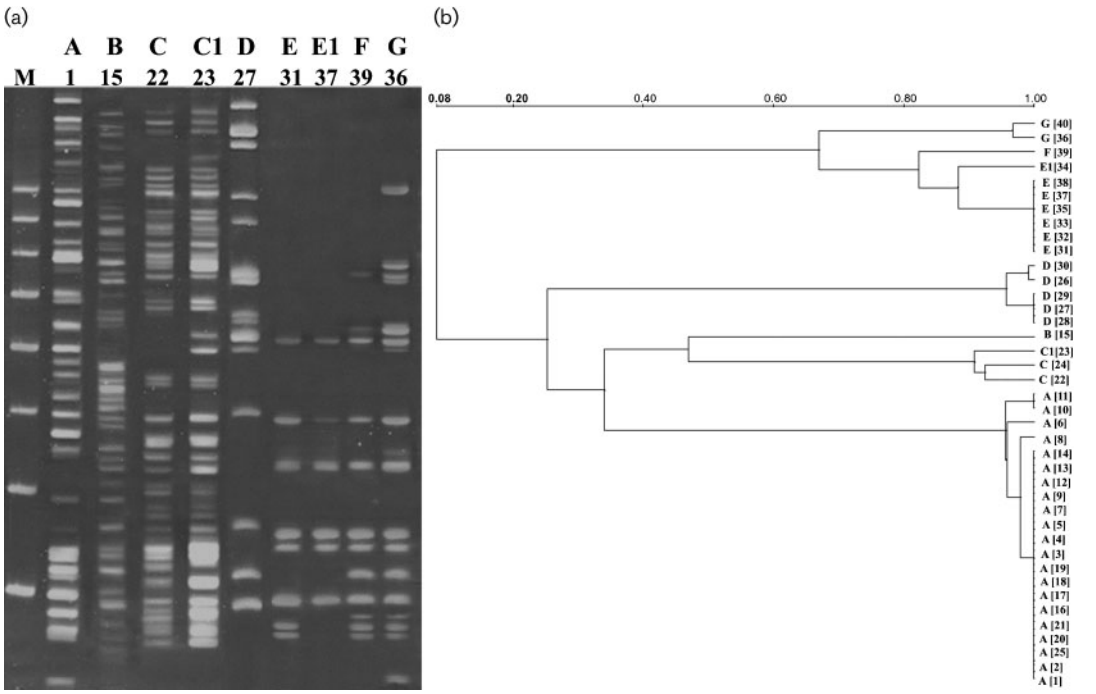

Fig. 3. Representative samples of PCR-MP fingerprints (a) and the UPGMA dendrogram (b) obtained for *T. interdigitale* isolates following *Hin*dIII restriction. Lane M, molecular size markers (1000, 900, 800, 700, 600, 500, 400, 300 and 200 bp). The number of isolates and PCR-MP types (assigned as in Table 1) are given above each lane. Electrophoresis of the DNA amplicons was carried out on a 6 % polyacrylamide gel.

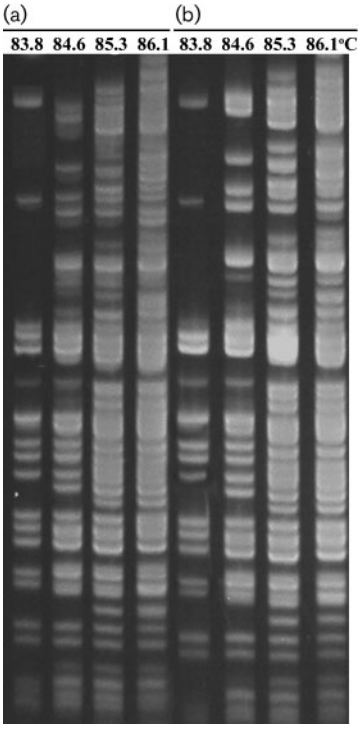

Fig. 4. Comparison of PCR-MP results for two *M. canis* isolates, isolate no. 3 (a) and isolate no. 14 (b), over a denaturation temperature gradient (83.8, 84.6, 85.3 and 86.1 °C). Electrophoresis of the DNA amplicons was carried out on a 6 % polyacrylamide gel.

infection, for example whether the origins of the fungal infection are from the patient's family members or from public facilities such as schools, swimming pools or military establishments. They can also help to find strain types that possess enhanced infectivity or invasiveness or are more virulent. They could also provide the answer to the question of different susceptibilities of keratinized structures to dermatophyte infections, for example to determine whether certain strains are responsible for onychomycosis rather than a skin infection (Macura, 2006). For chronic dermatophytoses, typing methods can answer the question of whether consecutive infections represent the acquisition of a new strain or reactivation of a strain type that has not been adequately cleared, which would reveal the lack of success of an applied therapy. Analyses of successive isolates can also reveal the impact of treatment on a population of isolates, and can provide an estimate of the efficacy of existing therapies and the adequacy of infection control measures, and can establish a link between treatment and changes in antimicrobial susceptibility patterns. The treatment of dermatophytoses can last for several months, increasing the potential for acquisition of resistance to drugs. Typing methods will allow better characterization of infecting strains and monitoring of their occurrence and distribution. However, one of the most important preliminary investigations in the molecular epidemiology of dermatophytes is to determine whether infections are caused by one or more strains.

One of the most popular typing methods used for differentiation of dermatophytes is RAPD (Baeza & Giannini, 2004; Baeza *et al.*, 2006; Kac *et al.*, 1999; Kaszubiak *et al.* 2004). However, one of the main drawbacks of this method is its low reproducibility, which affects its reliability (Ellsworth *et al.*, 1993).

In this study, PCR-MP was used for the first time to our knowledge as an alternative simple method for intraspecies differentiation of dermatophytes. This technique has been successfully used previously for the differentiation of bacterial strains of *E. coli* (Krawczyk *et al.*, 2006), *Enterococcus faecium* (Krawczyk *et al.*, 2007a) and *Staphylococcus aureus* (Krawczyk *et al.*, 2007b). The method used for analysis was optimized according to the procedure applied in earlier studies (Krawczyk *et al.*, 2006, 2007a, b). We chose restriction systems and appropriate denaturation temperatures to obtain a high differentiation power for the PCR-MP method.

We distinguished five types (A–E) for the *T. rubrum* isolates (four genotypes among ten clinical isolates from Poland and one reference strain). We obtained similar results for RAPD, but the PCR-MP method additionally distinguished one subtype (A1). For PCR-MP analysis of *T. interdigitale* isolates, we determined three genotypes (A, C and D) among the 29 clinical isolates from Lodz, Poland, and three genotypes (E, F and G) among the ten isolates from Copenhagen, Denmark. The reference strain displayed a different genotype (B). RAPD revealed a lower discriminatory power, determining only four genotypes.

We did not distinguish many genotypes among the *T. interdigitale* isolates. This probably results from the low frequency of changes in DNA among populations of strains originating from the same region. Some zoophilic strains of *T. interdigitale* belonged to the same genotype as anthropophilic strains. However, PCR-MP analysis showed that the zoophilic *T. interdigitale* strains originating from Poland were different from the zoophilic strains originating from Denmark. These results suggest that this method can be successfully applied to studies of the epidemic distribution of *T. interdigitale* strains and could be used as an alternative method to RAPD in epidemiological studies. The advantage of PCR-MP is its better reproducibility and stability. No specialized equipment is needed and the procedures are simple. These two techniques (PCR-MP and RAPD) could be used together as methods of choice for screening isolates of *T. rubrum* and *T. interdigitale.*

Despite the fact that the isolates of *M. canis* were tested with four different restriction enzymes for PCR-MP analysis, we did not observe any differentiation among the clinical isolates and the reference strain. These results were confirmed using RAPD, using three sets of primers

that have been used previously for dermatophyte analysis (Baeza *et al.*, 2006; Gräser *et al.*, 2000; Kac *et al.*, 1999). This suggests that the lack of intraspecies polymorphism might be due to the low clonal diversity of the analysed species caused by genetic stability of the *M. canis* genome. Results confirming this hypothesis have been shown by Sharma *et al.* (2007), who discovered over-representation among human isolates of one genotype, which had a higher degree of virulence and had a pandemic distribution, and by other authors who have reported low variability among epidemiologically unrelated strains from cats, dogs and humans, despite their morphological diversity (Brilhante *et al.*, 2005; Faggi *et al.*, 2001).

In conclusion, PCR-MP analysis is a simple method with a high discriminatory power and low cost, and may be very useful for epidemiological studies of dermatophytes, especially *T. rubrum* and *T. interdigitale*.

ACKNOWLEDGEMENTS

This work was supported by grants (N404 078 31/3454 and N401 0728 33/0728) from The State Committee for Scientific Research (Poland). This research was also supported by the European Union within the European Social Fund in the framework of the project 'InnoDoktorant – Scholarships for PhD students, I edition'. We are grateful to Birgit Brandt from Department of Bacteriology, Mycology and Parasitology of the Statens Serum Institute in Denmark.

REFERENCES

Abdel-Rahman, S. M. (2008). Strain differentiation of dermatophytes. *Mycopathologia* **166**, 319–333.

Baeza, L. C. & Giannini, M. J. (2004). Strain differentiation of *Trichophyton rubrum* by random amplification of polymorphic DNA (RAPD). *Rev Inst Med Trop Sao Paulo* **46**, 339–341.

Baeza, L. C., Matsumoto, M. T., Almeida, A. M. F. & Mendes-Gianini, M. J. S. (2006). Strain differentiation of *Trichophyton rubrum* by randomly amplified polymorphic DNA and analysis of rDNA nontranscribed spacer. *J Med Microbiol* **55**, 429–436.

Brilhante, R. S., Cordeiro, R. A., Medrano, D. J., Monteiro, A. J., Sidrim, J. J. & Rocha, M. F. (2005). Antifungal susceptibility and genotypical pattern of *Microsporum canis* strains. *Can J Microbiol* **51**, 507–510.

de Assis Santos, D., de Carvalho Araújo, R. A., Kohler, L. M., Machado-Pinto, J., Hamdan, J. S. & Cisalpino, P. S. (2007). Molecular typing and antifungal susceptibility of *Trichophyton rubrum* isolates from patients with onychomycosis pre- and post-treatment. *Int J Antimicrob Agents* **29**, 563–569.

Dobrowolska, A., Staczek, P., Kaszuba, A. & Kozłowska, M. (2006). PCR-RFLP analysis of the dermatophytes isolated from patients in Central Poland. *J Dermatol Sci* **42**, 71–74.

Ellsworth, D. L., Rittenhouse, K. D. & Honeycutt, R. L. (1993). Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **14**, 214–217.

Faggi, E., Pini, G., Campisi, E., Bertellini, C., Difonzo, E. & Mancianti, F. (2001). Application of PCR to distinguish common species of dermatophytes. *J Clin Microbiol* **39**, 3382–3385.

Gräser, Y., Kuijpers, A. F. A., Presber, W. & Hoog, G. S. (2000). Molecular taxonomy of the *Trichophyton rubrum* complex. *J Clin Microbiol* **38**, 3329–3336.

Jackson, C. J., Barton, R. C., Kelly, S. L. & Evans, E. (2000). Strain identification of *Trichophyton rubrum* by specific amplification of subrepeat elements in the ribosomal DNA nontranscribed spacer. *J Clin Microbiol* **38**, 4527–4534.

Kac, G., Bougnoux, M. E., Feulhade De Chauvin, M., Sene, S. & Derouin, F. (1999). Genetic diversity among *Trichophyton mentagrophytes* isolates using random amplified polymorphic DNA method. *Br J Dermatol* **140**, 839–844.

Kamiya, A., Kikuchi, A., Tomita, Y. & Kanbe, T. (2004). PCR and PCR-RFLP techniques targeting the DNA topoisomerase II gene for rapid clinical diagnosis of the etiologic agent of dermatophytosis. *J Dermatol Sci* **34**, 35–38.

Kaszubiak, A., Klein, S., de Hoog, G. S. & Gräser, Y. (2004). Population structure and evolutionary origins of *Microsporum canis*, *M. ferrugineum* and *M. audouinii*. *Infect Genet Evol* **4**, 179–186.

Krawczyk, B., Samet, A., Leibner, J., Śledzińska, A. & Kur, J. (2006). Evaluation of a PCR melting profile technique for bacterial strain differentiation. *J Clin Microbiol* **44**, 2327–2332.

Krawczyk, B., Leibner, J., Stojowska, K., Bronk, M., Samet, A. & Kur, J. (2007a). PCR melting profile method for genotyping of vancomycin-resistant *Enterococcus faecium* isolates from hematological unit patients. *Pol J Microbiol* **56**, 65–70.

Krawczyk, B., Leibner, J., Barańska-Rybak, W., Samet, A., Nowicki, R. & Kur, J. (2007b). ADSRRS-fingerprinting and PCR MP techniques for studies of intraspecies genetic relatedness in *Staphylococcus aureus*. *J Microbiol Methods* **71**, 114–122.

Liu, D., Coloe, S., Baird, R. & Pederson, J. (2000). Rapid mini-preparation of fungal DNA for PCR. *J Clin Microbiol* **38**, 471.

Macura, A. B. (2006). Nail susceptibility to ex vivo experimental fungal infections. *Mikologia Lekarska* **13**, 255–260.

Masny, A. & Płucienniczak, A. (2003). Ligation mediated PCR performed at low denaturation temperatures – PCR melting profiles. *Nucleic Acids Res* **31**, e114.

Mochizuki, T., Ishizaki, H., Barton, R. C., Moore, M. K., Jackson, C. J., Kelly, S. L. & Evans, E. G. V. (2003). Restriction fragment length polymorphism analysis of ribosomal DNA intergenic regions is useful for differentiating strains of *Trichophyton mentagrophytes*. *J Clin Microbiol* **41**, 4583–4588.

Sharma, R., de Hoog, S., Presber, W. & Gräser, Y. (2007). A virulent genotype of *Microsporum canis* is responsible for the majority of human infections. *J Med Microbiol* **56**, 1377–1385.

Weitzman, I. & Summerbell, R. (1995). The dermatophytes. *Clin Microbiol Rev* **8**, 240–259.

Yazdanparast, A., Jackson, C. J., Barton, R. C. & Evans, E. G. V. (2003). Molecular strain typing of *Trichophyton rubrum* indicates multiple strain involvement in onychomycosis. *Br J Dermatol* **148**, 51–54.

Yu, J., Wan, Z., Chen, W., Wang, W. & Li, R. (2004). Molecular typing study of the *Microsporum canis* strains isolated from an outbreak of tinea capitis in a school. *Mycopathologia* **157**, 37–41.