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Application of Microsatellite-Primed PCR (MSP-PCR) and PCR Melting Profile (PCR-MP) Method for Intraspecies Differentiation of Dermatophytes

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Abstract

In this study, two PCR-based methods (MSP-PCR and PCR-MP) were compared for their abilities to identify intraspecies variations of 23 isolates of *Trichophyton rubrum*, 78 isolates of *Trichophyton interdigitale* and 22 isolates of *Microsporum canis*, obtained mainly from patients in Łódź city. The results allowed to distinguish four types (containing two subtypes) characteristic for *T. interdigitale* and three types characteristic for *T. rubrum* using PCR-MP method. Analysis conducted using MSP-PCR with $(GACA)_4$ primer revealed four types for *T. rubrum* and three types (containing one subtype) for *T. interdigitale* and with $(GTG)_5$ primer showed two types (containing one subtype) for *T. rubrum* and six types (containing one subtype) for *T. interdigitale*. No differentiation was observed for the *M. canis* isolates with either method.

Key words: dermatophytes, PCR-MP, MSP-PCR

Introduction

Dermatophytes are keratinophilic and keratinolytic fungi responsible for infections of skin and its products such as hair and nails. Among this group of fungi three genera *Trichophyton*, *Microsporum* and *Epidermophyton* are identified, which contain species pathogenic for human and animals. Dermatophyte infections pose a significant dermatological and epidemiological problem. Contemporary conditions of life increase probability of the occurrence of conditions which are favorable for the development of such infections. This finds confirmation by the increasing number of fungal infections, including dermatophytoses within last years. Dermatophyte infections are reported not only in particular age groups, such as children and elderly people, but also in social and professional ones. Because of that, skin infections caused by dermatophytes are currently recognized as diseases of civilization which pose a serious public health problem.

Epidemiological analysis conducted in different regions of Poland indicates that *tinea pedis* and *onychomycoses* are the most common infections caused in mainly by dermatophytes (93% of *onychomycosis* cases and 95% of *tinea pedis*) (Hryncewicz-Gwóźdź *et al.*, 2005). The most often isolated species in this kind of dermatophytoses are *T. rubrum* and *Trichophyton mentagrophytes* and in the case of *tinea corporis T. mentagrophytes* and *M. canis* are predominant.

Due to the limited knowledge on molecular biology, genetics, genomics, and proteomics of these pathogenic fungi, until recently there were no methodological approaches for intraspecies dermatophyte differentiation which is the sine qua non to determine an infection source and epidemiological relationships. In the current literature frequent opinions are showing up that the effective therapy requires precise diagnosis of etiological agent and the increasing number of dermatophyte infections will force the necessity to elaborate common epidemiological analysis schemes (Hryncewicz-Gwóźdź *et al.*, 2005).

Many molecular techniques have been developed to provide rapid and accurate alternatives for traditional dermatophyte identification. These methods include

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Table I
Clinical profile of patients from whom identified species of dermatophytes were isolated

Species	Total no. of isolates	No. (% of samples) of isolates from:			No. (% of isolates) from patients:						
					Sex			Age (year)			
		Hair	Skin	Nail	Female	Male	ND	≤ 18	> 18 ≤ 50	>50	*ND*
Trichophyton rubrum	23	1 (4%)	6 (26%)	16 (70%)	13 (52%)	12 (48%)	0 (0%)	1 (4%)	9 (36%)	15 (60%)	0 (0%)
Trichophyton interdigitale	78	6 (7.74%)	4 (5.12%)	68 (87.17%)	25 (32.89%)	23 (30.27%)	28 (36.84%)	3 (3.95%)	27 (35.53%)	18 (23.68%)	28 (36.84%)
Microsporum canis	22	4 (18.18%)	4 (18.18%)	14 (63.94%)	8 (36.36%)	14 (63.64%)	0 (0%)	6 (27.27%)	9 (40.91%)	7 (31.82%)	0 (0%)

ND – No Data

gene-specific PCR (Kanbe, 2008; Jackson *et al*., 1999; 2000), RFLP analysis, sequencing of the large and small subunit rRNA gene (Mochizuki *et al*., 2003; Rezaei-Matehkolaei *et al*., 2012; Dobrowolska *et al*., 2006), chitin synthase-encoding gene (Kano *et al*., 1999; 2001; 2002a), topoisomerase II gene (Kanbe *et al*., 2003a; 2003b) PCR fingerprinting (Faggi *et al*., 2001), Single-Step PCR (Shehata *et al*., 2008). Sequencing of the internal transcribed spacer (ITS) regions (Gräser *et al*., 2008) has proved to be a useful method for phylogenetic analysis and identification of dermatophytes.

The objective of the study was the application of the PCR melting profile (PCR-MP) technique and microsatellite-primed PCR was performed using two sets of primers – $(GACA)_4$ and $(GTG)_5$, for the purpose of strain typing of dermatophytes isolated mainly from patients in Łódź city, Poland.

Experimental

Material and Methods

Fungal strains. Twenty three isolates of *T. rubrum*, 78 of *T. interdigitale* and 22 of *M. canis* were used in this study (Table I). Samples were collected from hair, skin, toenails and fingernails. Standard mycological identification according to morphology of fungal colonies was performed at the 2nd Department of Dermatology, Pediatric Dermatology and Oncology Clinic, Medical University of Łódź. Traditional identification was confirmed by PCR-RFLP identification targeting the ITS1-5.8S-ITS2/*Hinf*I region (Mochizuki *et al*., 2003). Reference strains: *T. rubrum* (120358), *T. mentagrophytes* (120357) and *M. canis* (113480) used in this study originated from the Dutch CBS (Centraalbureau voor Schimmelcultures) collection.

DNA extraction. Total cellular DNA was extracted from a mycelium cultured on Sabouraud agar slants by 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 2 h. After the addition of 210 µl 3 M sodium acetate, the homogenate was centrifuged at 10 000 × 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 for 1 h at 36°C. The samples were then precipitated using 3 volumes of 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 30 µl Tris-EDTA buffer, and 1 µl of the resulting solution was used as template in the following PCR.

Microsatellite-primed PCR (MSP-PCR) using $(GACA)_4$ and $(GTG)_5$ oligonucleotides. MSP-PCR amplification was performed using $(GACA)_4$ or $(GTG)_5$ primers (Table II). Amplifications were performed with approximately 20 ng DNA in 25 µl reaction mixtures containing 12,5 µl 2 × PCR Master Mix Start-Warm (A&A BIOTECHNOLOGY) and 0,25 µl primer (100 µM). PCRs were performed in Thermal Cycler C1000™ (BIORAD) as follows: initial denaturation at 95°C for 5 min, 40 cycles at 93°C for 60 s, 50°C for 60 s and 72°C for 60 s; and final cycle at 72°C for 6 min. All

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

Name	Sequence (5'-3')
PCR MP	
Restrictionenzyme	**Helper oligonucleotide**
*Bam*HI	GATCGTCGACGTTGG
	Ligated oligonucleotide
PowieTm	CTCACTCTCACCAACAACGTCGAC
MP primer	
MP BamHI	CTCACTCTCACCAACGTCGACGATCC
MSP-PCR	
$(GACA)_4$	GACAGACAGACAGACA
$(GTG)_5$	GTGGTGGTGGTGGTG

amplification products were analyzed by electrophoresis of 5 µl samples on 1.2% agarose gels (Shehata *et al*., 2008; Roque *et al*., 2006; Spesso *et al*., 2013).

PCR-MP analysis. PCR-MP procedure was optimized according to the method described for dermatophytes (Leibner-Ciszak *et al*., 2010) with modification. DNA was digested with *Bam*HI (10 U/µl) restriction enzyme (ThermoScientific). Digestion reactions were performed under uniform conditions: approximately 200 ng of DNA sample was added to 2.5 µl of Buffer BamHI (10 × concentrated, ThermoScientific), and 0.5 µl (5 U) of the endonuclease in a total volume of 25 µl. After incubation at 37°C for 3 h, the following ligation mix was added: 2 µl of two oligonucleotides (20 pmol of each) that formed an adapter (Table II), 2.5 µl of ligation buffer [400 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM dithiothreitol, 5 mM ATP; ThermoScientific], and 0.1 µl of T4 DNA ligase (0.5 U; ThermoScientific). The samples were then incubated at 22°C overnight. 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], 0.5 µl dNTP mix (200 µM each), 0.5 µl (1 U) *Shark* polymerase (DNA Gdańsk) and 25 pmol MP-B primer (Table II).

The denaturation temperature was determined by specific optimization experiments with DNAs of reference strain and the number of clinical isolates of *T. rubrum*, *T. interdigitale* and *M. canis* using a gradient thermal cycler (Thermal Cycler C1000™, BIORAD) within a gradient range of 80–84°C. The PCRs were performed as follows: 7 min at 72°C; initial denaturation for 90 s over a gradient 80.0–84°C, 24 cycles of denaturation for 1 min at a gradient 80.0–84°C, annealing and elongation step at 72°C for 2 min 15 s, and final elongation at 72°C for 5 min. PCRs for *T. rubrum*, *T. interdigitale* and *M. canis* were performed as described above, using the established optimal denaturation temperature 82.8°C, 82.4°C and 82.6°C, respectively. All analyses were performed in triplicate, and PCRs were performed using two different thermal cyclers: Thermal Cycler C1000™ (BIORAD) and Labcycler 48 Gradient (SensoQuest Biomedical Electronics). Electrophoresis of all PCR products was performed on 6% polyacrylamide gels.

Data 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. Cluster analysis was performed using a Versa Doc Imaging System version 1000 (BIORAD) with Quantity One software (version 4.4.0). DNA relatedness was calculated by the band-based Dice coefficient with a setting of 2% band tolerance using the unweighted pair group method with mathematical averaging (UPGMA).

Results

Clinical profiles. Clinical data about patients from which dermatophytes were identified, are summarized in Table I. The anthropohilic *T. interdigitale* strains: 48 isolates originating from Łódź and 28 isolates originating from Rzeszów, were collected mainly from female patients above 18 years in age. *T. rubrum* isolates originating from Łódź, were obtained from male and female at 1:1 ratio, mostly the oldest (> 50 years) patients. The third species originating from Łódź was *M. canis* isolated mainly from male patients between 18 and 50 years old.

$(GACA)_4$ and $(GTG)_5$ typing. All isolates of *T. rubrum*, *T. interdigitale* and *M. canis* were analysed by MSP-PCR method using $(GACA)_4$ or $(GTG)_5$ microsatellite primer. Among 23 isolates of *T. rubrum*, four $(GACA)_4$ genotypes were distinguished (Tr/A-Tr/D) (Fig. 1A, Table III) and two $(GTG)_5$ genotypes (Tr/a and Tr/b) (Fig. 2A, Table III) . In case of $(GACA)_4$ typing, Tr/A genotype was markedly predominant and was characteristic for twenty *T. rubrum* isolates originating from Łódź city, Poland. Genotypes Tr/B, Tr/C and Tr/D were represented by single isolates (Table III). Tr/a genotype was predominant in case of $(GTG)_5$ typing of *T. rubrum*, and characteristic for twenty one isolates. Tr/b genotype (containing one subtype Tr/b1) was represented only by one isolate.

All 50 isolates of *T. interdigitale* originating from Łódź, and 28 isolates of *T. interdigitale* originating

Table III
Genotypes of dermatophytes obtained by MSP-PCR and PCR-MP method

Species of dermatophyte	Name (no.) of genotypes identified		
	$(GACA)_4$	$(GTG)_5$	PCR-MP/ *Bam*HI
T. rubrum, 23 isolates	Tr/A (20)	Tr/a (21)	Tr/I (20)
	Tr/B (1)	Tr/b (1)	Tr/II (1)
	Tr/C (1)	Tr/b1(1)	Tr/III (2)
	Tr/D (1)		
T. interdigitale, 78 isolates	Ti/A (73)	Ti/a (67)	Ti/I (68)
	Ti/A1 (2)	Ti/a1(4)	Ti/Ia (2)
	Ti/A2 (1)	Ti/b (3)	Ti/Ib (2)
	Ti/B (1)	Ti/c (1)	Ti/II (1)
	Ti/C (1)	Ti/d (1)	Ti/III (4)
		Ti/e (1)	Ti/IV (1)
		Ti/f (1)	
M. canis, 22 isolates	Mc/A (22)	Mc/a (22)	Mc/I (22)

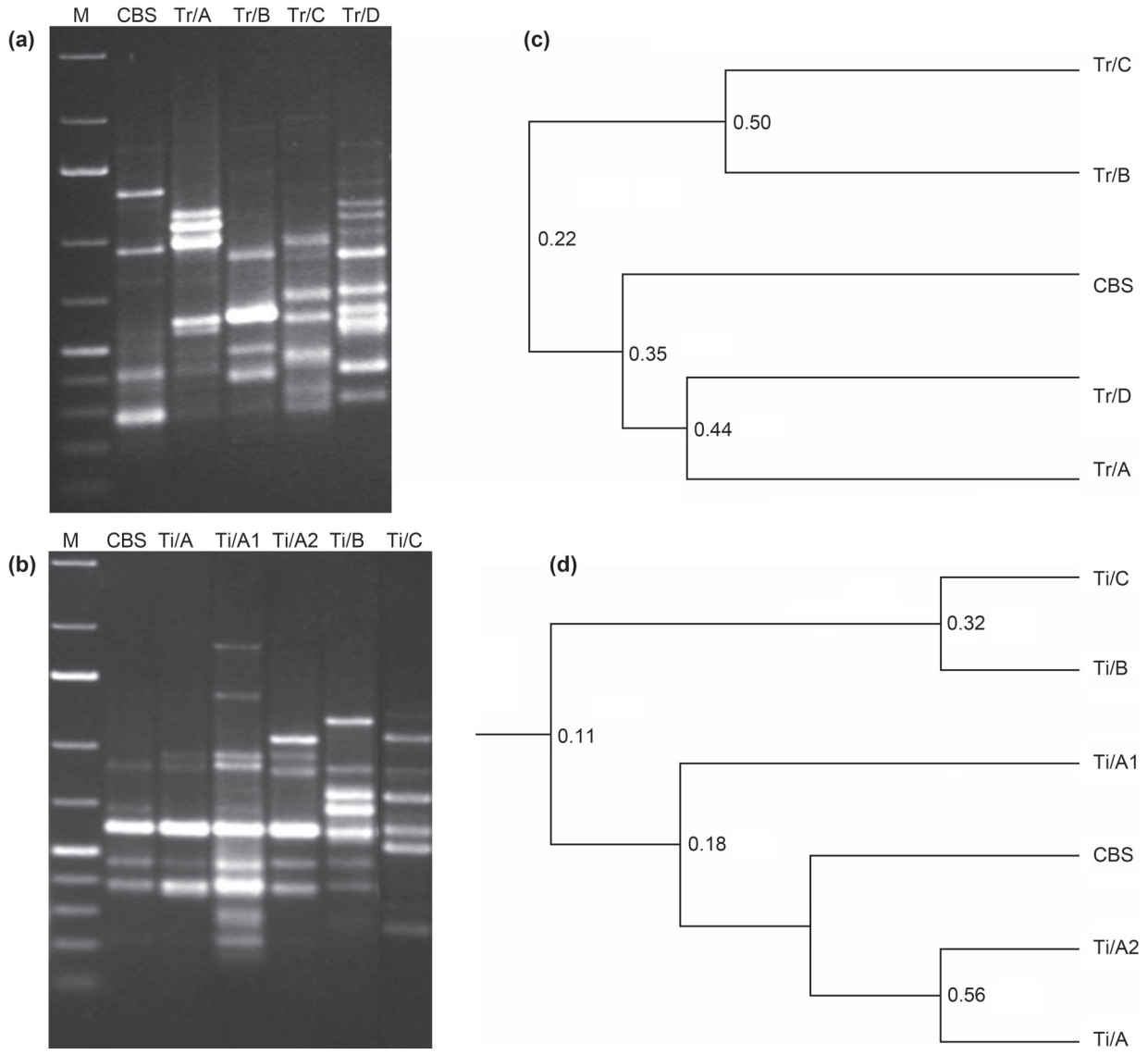

Fig. 1. $(GACA)_4$ genotyping of *T. rubrum* **(a)** and *T. interdigitale* **(b)** isolates using MSP-PCR method and unweighted pair group method with arithmetic mean (UPGMA) dendrogram **(c, d)**. Lane M, molecular size marker (2000, 1500, 1000, 700, 500, 400, 300, 200, 75 bp). Electrophoresis of the DNA amplicons was carried out on 1.2 % agarose gel (Ciesielska A.).

from Rzeszów, were classified into three $(GACA)_4$ genetic groups (Ti/A-Ti/C) (Fig. 1A, Table III) and six $(GTG)_5$ groups (Ti/a-Ti/f) (Fig. 2B, Table III). $(GACA)_4$ Ti/A genotype (containing two subtypes Ti/A1 and Ti/A2) was predominant and represented by 76 isolates (49 isolates originating from Łódź and 27 isolates from Rzeszów), Ti/B and Ti/C genotypes were represented by single isolates, originating from Rzeszów and Łódź, respectively. In case of $(GTG)_5$ typing of *T. interdigitale*, Ti/a genotype (containing one subtype Ti/a1) was predominant and characteristic for 71 isolates (43 isolates originating from Łódź and 28 originating from Rzeszów), Ti/b genotype was represented by three isolates originating from Łódź, and one isolate of *T. interdigitale* was assigned to Ti/c, Ti/d, Ti/e genotype and Ti/f, originating from Łódź, respectively.

Among 22 *M. canis* isolates, single $(GACA)_4$ and $(GTG)_5$ genotypes were distinguished (Table III).

PCR-MP analysis. All isolates of *T. rubrum*, *T. interdigitale* and *M. canis* were analyzed using *Bam*HI restriction endonuclease which gave PCR-MP fingerprints for the examined isolates (Fig. 3A, 3B, Table III). Among 23 isolates of *T. rubrum*, we distinguished three (Tr/I-Tr/III) genotypes (Fig. 3A, Table III). Genotype Tr/I was markedly predominant and represented by twenty isolates. The remaining isolates were classified as two different genotypes (Tr/II and Tr/III), specific for one and two isolates, respectively. In the case of the *T. interdigitale* isolates, PCR-MP analysis revealed four genotypes (Ti/I-Ti/IV) (Fig. 3B, Table III). Genotype Ti/I was predominant and contained 40 isolates originating from Łódź, and 28 isolates originating from Rzeszów. The PCR-MP technique was able to differen-

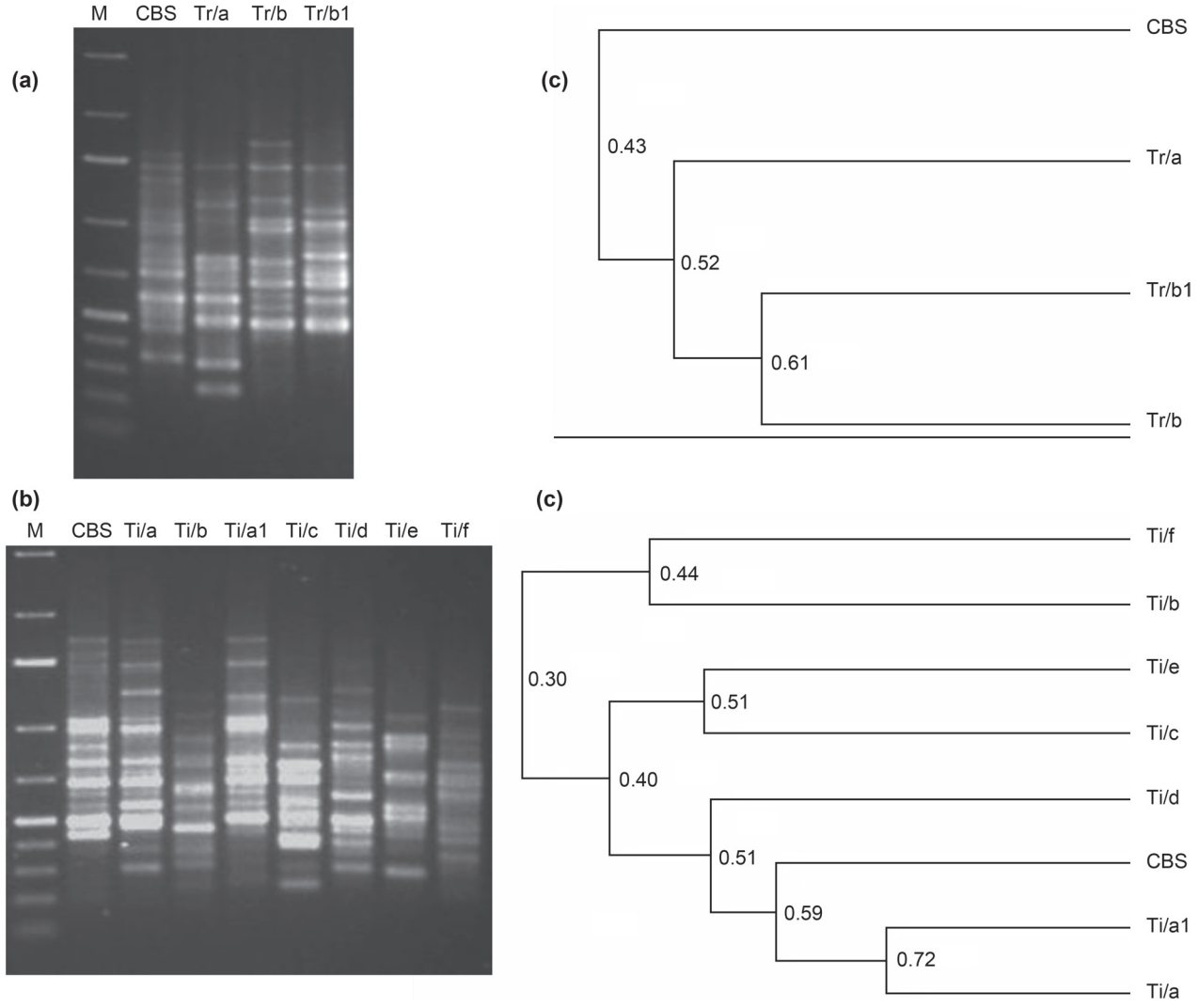

Fig. 2. $(GTG)_5$ genotyping of *T. rubrum* **(a)** and *T. interdigitale* **(b)** isolates using MSP-PCR method and unweighted pair group method with arithmetic mean (UPGMA) dendrogram **(c, d)**. Lane M, molecular size marker (2000, 1500, 1000, 700, 500, 400, 300, 200, 75 bp). Electrophoresis of the DNA amplicons was carried out on 1.2 % agarose gel (Ciesielska A.).

tiate two subtypes (Ti/Ia and Ti/Ib) among genotype Ti/I (Fig. 3B). Genotype Ti/III was characteristic for four isolates, and single isolates of *T. interdigitale* were assigned to genotypes Ti/II and Ti/IV, respectively. For 22 isolates of *M. canis* only one characteristic pattern (Mc/I) was obtained by PCR-MP analysis (Table III)

Discussion

Dermatophyte superficial infections are extremely frequent worldwide and their geographical distribution have changed in the last decades as a consequence of several factors, such as migration of population, economic development, industrialization, antibiotic therapy, changes in climat conditions. Epidemiological studies conducted in different regions of Poland also indicate changes in percentage of dermatophyte infections within years (Hryncewicz-Gwóźdź *et al*., 2005). In the years 1988–1992 Baran and Szepietowski [20] updated epidemiological map of Poland, and have demonstrated an advantage in the incidence of anthropohilic dermatophyes (64.3%) over zoophilic ones (35%). Previous studies (Hryncewicz-Gwóźdź *et al*., 2005; Wroński and Nowicki, 2000; Lange, 2002) conducted in Poland (1952–1957 and 1974–1979) showed a distinct advantage of zoophilic dermatophytes, representing 69,9% of the total dermatophytes over antropohilic ones (30%). The results of both analyses demonstrated how large epidemiological changes took place in relation to the incidence of dermatophyte infections in Poland during the last 30–40 years. In Central Poland, over the years 1987–1996, *T. rubrum* was the main etiological agent (34.15%) and the second most common dermatophyte was *T. mentagrophytes* (33.19%) (Kaszuba, 1997). The authors pointed out an interesting observation regarding significant change in the isolation of *T. rubrum* and *T. mentagrophytes*. In case of

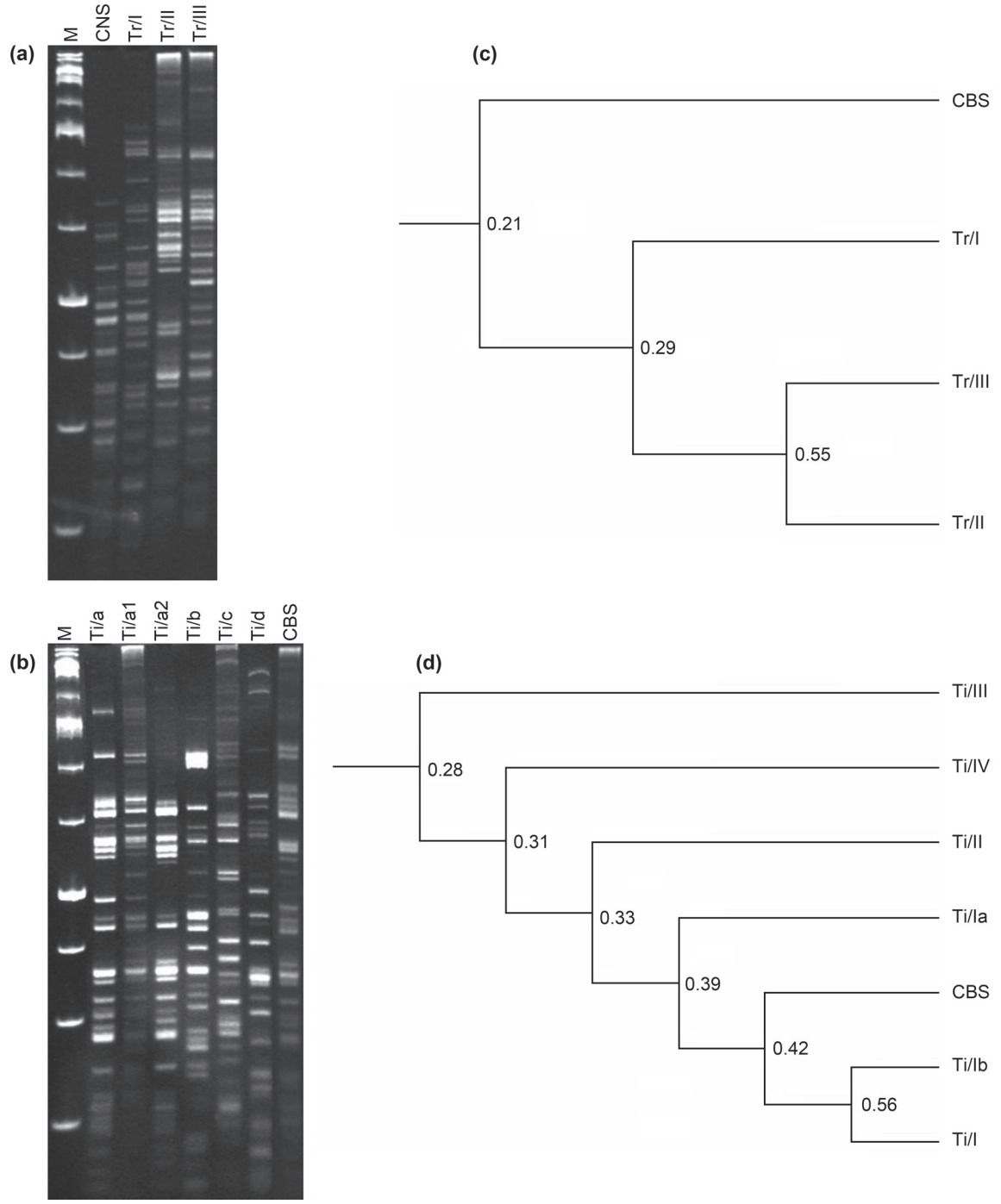

Fig. 3. PCR-MP profiles of *T. rubrum* **(a)** and *T. interdigitale* **(b)** isolates using *Bam*HI restriction enzyme and unweighted pair group method with arithmetic mean (UPGMA) dendrogram **(c, d)**. Lane M, molecular size marker (2000, 1500, 1000, 700, 500, 400, 300, 200, 75 bp). Electrophoresis of the DNA amplicons was carried out on 6 % polyacrylamide gel (Ciesielska A.).

T. rubrum the number of isolation has increased from 25% to 34%, which was accompanied by the decrease of infections caused by *T. mentagrophytes* from 50% to 33% (Kaszuba, 1997).

Molecular methods used in diagnostics and epidemiology of pathogenic fungi infections are based on the analysis of polymorphisms of particular genetic markers, therefore they require a search for unique nucleotide sequences in the DNA, specific for genus, species, and even strain. In eukaryotic genomes there are fragments which undergo fast evolutionary changes and thus they are highly diverse among individual species. Search for such sequences gives a chance to develop new genetic markers. Analysis of the scientific literature shows that in the recent 7–8 years the attempts were made in different countries and laboratories, including ours, to utilize specific sequences of genomic DNA as molecular markers in identification and differentiation of individual genera and species of dermatophytes. Moreover, for the purpose of investigation of derma-

tophyte infection sources and transmission pathways in populations of humans and animals, it is necessary to have available techniques allowing deep genetic differentiation of strains within species, therefore enabling prompt and reliable identification of individual clones. Until now, in case of some dermatophyte species such methods were not available. Molecular typing methods for dermatophytes can provide crucial insights into their epidemiology and pathogenecity. They can also help to characterize infecting strains and monitor their occurrence and distribution. Finally, the most important investigations in molecular epidemiology of dermatophytes is to determine whether infections are caused by one or more strains.

In this study, we demonstrated the application of PCRs using single microsatellite primers, $(GACA)_4$ and $(GTG)_5$ and PCR melting profile as simple methods which may be useful for intraspecies differentiation of dermatophytes. Microsatellite-primed PCR using $(GACA)_4$ and $(GTG)_5$ were previously used mainly for species identification of dermatophytes (Shehata *et al*., 2008; Roque *et al*., 2006; Spesso *et al*., 2013) but not as typing methods. The authors showed only few cases of instraspecies variations within *T. mentagrophytes* strains using $(GACA)_4$ primer (Shehata *et al*., 2008). In this work, MSP-PCR was used for molecular typing of *T. rubrum*, *T. interdigitale* (former *T. mentagrophytes* var. *interdigitale* (Gräser *et al*., 2008) and *M. canis* strains isolated from patients in Łódź and Rzeszów cities. The *T. rubrum* isolates from skin, hair and nails exhibited four $(GACA)_4$ genotypes (Tr/A-Tr/D) and two $(GTG)_5$ genotypes, the *T. interdigitale* isolates showed three $(GACA)_4$ genotypes (Ti/A-Ti/CC) and six $(GTG)_5$ genotypes and finally individual $(GACA)_4$ and $(GTG)_5$ patterns were obtained for *M. canis* isolates. The reference strains of all analyzed species of dermatophytes individual genotypes.

MSP-PCR using $(GACA)_4$ and $(GTG)_5$ primers was found to be useful for the differentiation of *T. rubrum* and *T. interdigitale* isolates. On the other hand, no clonal diversity was observed between *M. canis* isolates. However, the degree of discrimination of particular species by means of MSP-PCR depends highly on the primers used. In case of *T. rubrum* isolates, PCR fingerprinting with $(GACA)_4$ primer enabled higher level of discrimination than $(GTG)_5$ primer On the other hand, $(GTG)_5$ primer showed a higher differentiation power than $(GACA)_4$ primer in case of *T. interdigitale* isolates. The absence of intraspecies variation within *M. canis* isolates, revealed by this method may suggest that strain specific variation of analyzed species is not located in the $(GACA)_4$ or $(GTG)_5$ region. However, our $(GACA)_4$ typing results are in agreement with the results obtained by Faggi *et al.* (2001), and Shehata *et al.* (2008) who showed no intraspecies variation within analyzed *M. canis* isolates by single-step PCR using $(GACA)_4$ primer.

PCR-MP method was used as an alternative simple method for intraspecies differentiation of analyzed dermatophytes. This technique has been successfully used for distinguishing bacterial strains such as *Escherichia coli*, *Staphylococcus aureus* and also was applied by our group for differentiation of dermatophytes (Krawczyk *et al*., 2006; 2007). The method used for analysis was optimized according to the procedure described in earlier study (Leibner-Ciszak *et al*., 2010). We chose the restriction enzyme and appropriate denaturation temperature to obtain high differentiation power for the PCR-MP method. Four types for *T. rubrum* isolates (one genotype for reference strain), five types for *T. interdigitale* (one genotype for reference strain) and one for *M. canis* isolates were distinguished. In case of *T. rubrum* isolates PCR-MP revealed lower discriminatory power than MSP-PCR using $(GACA)_4$ and $(GTG)_5$ primers. PCR-MP analysis of *T. interdigitale* isolates showed also lower reproducibility than $(GTG)_5$ typing, but on the other hand, it yielded more genotypes than $(GACA)_4$ analysis. Similar results were also reported previously by our group (Leibner-Ciszak *et al*., 2010) (three PCR MP genotypes within *T. interdigitale* isolates and four genotypes specific for *T. rubrum* isolates) what may suggest low frequency of changes in DNA among population of analyzed isolates originating from the same region over the years.

Our results, using MSP-PCR and PCR-MP, showed that in each of the two methods one genotype – Ti/A, Ti/a, and Ti/I – was markedly predominant for the majority infection caused by *T. interdigitale* (Fig. 1B, 2B, 3B). The genotyping results suggest also that one strain of *T. rubrum* was identified continually (genotype Tr/A, Tr/a and Tr/I) (Fig. 1A, 2A, 3A) over the last years.

Molecular analysis of *M. canis* isolates showed once again the lack of clonal diversity. The reason may be due to the low discriminatory power of the markers employed or the genetic stability of this dermatophyte species as we suggested in our previous study (Dobrowolska *et al*., 2011). Faggi *et al.* (2001) also showed no $(GACA)_4$ intraspecies variability among analyzed *M. canis* strains what explained the low frequency of changes in DNA among populations of this zoophilic, dermatophyte species. This thesis can be confirmed by results obtained by Sharma *et al.* (2007) who observed overrepresentation of one genotype containing 74% of human and 23% of the animals strains which had a pandemic distribution. On the other hand, these data are in conflict with ISSR-PCR results obtained by Cano *et al.* (2005) who suggested that distribution of *M. canis* strains was restricted even to a single patients. However, Sharma *et al.* (2007) disputed the high discriminatory power of ISSR-PCR, explaining it its low reproducibility.

However, very promising results were published by Pasquetti *et al*. (2013) who showed the high resolving power and reproducibility of eight microsatellite markers (MS). The authors described MLMT method as a tool for intraspecies differentiation of *M. canis* strains originated from unrelated locations in 13 countries, suggesting also the usefulness of this system for detecting the source of *M. canis* infection and explaining the transmission pathway of this dermatophyte among human and animal population.

Despite previous reports (Shehata *et al*., 2008; Roque *et al*., 2006; Spesso *et al*., 2013) PCR utilizing $(GACA)_4$ and $(GTG)_5$ microsatellite primers showed mainly species specific patterns for investigated dermatophytes. The results of this study demonstrated that application of MSP-PCR with each of two primers allowed successful differentiation within clinical isolates of *T. rubrum* and *T. interdigitale* which had not been previously demonstrated.

In conclusion, the results of this work show that detection of intraspecies genetic relatedness in *T. rubrum* and *T. interdigitale* isolates by MSP-PCR using $(GACA)_4$ and $(GTG)_5$ primers, and PCR-MP method may be applied for epidemiological studies of this two species of dermatophytes.

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