

Microsatellite-primed PCR and random primer amplification polymorphic DNA for the identification and epidemiology of dermatophytes

M. F. Spesso · C. T. Nuncira · V. L. Burstein ·
D. T. Masih · M. D. Dib · L. S. Chiapello

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Abstract This study demonstrates the capacity of the one-step polymerase chain reaction (PCR) fingerprinting method using the microsatellite primers (GACA)₄ or (GTG)₅ (MSP-PCR) to identify six of the most frequent dermatophyte species causing cutaneous mycosis. PCR with (GACA)₄ was a suitable method to recognise *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum* and *Trichophyton interdigitale* among 82 Argentinian clinical isolates, producing the most simple and reproducible band profiles. In contrast, the identification of *Trichophyton mentagrophytes* and *Trichophyton tonsurans* was achieved using PCR with (GTG)₅. In this way, the sequential application of PCR using (GACA)₄ and (GTG)₅ allowed the successful typification of clinical isolates which had not been determined by mycological standard techniques. In this work, the intraspecies variability among 33 clinical isolates of *M. canis* was detected using random amplification of polymorphic DNA (RAPD-PCR) with the primers OPI-07 and OPK-20. The genetic variations in the isolates of *M. canis* were not associated with clinical features of lesions or pet ownership, but a geographical restriction of one genotype was determined with OPK-20, suggesting a clonal diversity related to different ecological niches in certain geographical areas. The results of this work

demonstrate that the detection of intraspecies polymorphisms in *M. canis* by RAPD-PCR may be applied in future molecular epidemiological studies to identify endemic strains, the route of infection in an outbreak or the coexistence of different strains in a single infection.

Introduction

Dermatophytes are the most common cause of fungal infections worldwide, with a global prevalence approaching 20 % [1]. Identification of the dermatophyte species is important for targeting treatment, infection control and epidemiological purposes [2]. Over recent years, changes have been reported in the epidemiology and ecological niches of common dermatophytes, as well as the emergence of new species or species more resistant to conventional antifungals [2, 3]. The three genera of these keratinophilic moulds, *Trichophyton*, *Microsporum* and *Epidermophyton*, are essentially typified based on their morphological features in cultures. However, the identification is complicated and time-consuming, due to morphological similarities, variability and the polymorphism shown by these fungi. In this sense, the introduction of a polymerase chain reaction (PCR)-based methodology can increase the specificity, simplicity, speed and, potentially, even reduce costs [4]. For studies on species identification and typing, PCR [5] and PCR fingerprinting (such as microsatellite-primed PCR (MSP-PCR) [6], random amplification of polymorphic DNA (RAPD) [7] and PCR-RFLP [8]) have all been applied. As the gold standard for the identification of atypical or difficult dermatophyte isolates, sequence analysis of the amplified internal transcribed spacer (ITS) is recommended in reference laboratories [9]. However, this method requires sequencing after amplification, resulting in a complex, expensive and time-consuming technique to apply for routine use in

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M. F. Spesso · V. L. Burstein · D. T. Masih · L. S. Chiapello (✉)
Centro de Investigaciones en Bioquímica Clínica e Inmunología,
CIBICI-CONICET, Departamento de Bioquímica Clínica,
Facultad de Ciencias Químicas, Universidad Nacional
de Córdoba, X5000HUA, Córdoba, Argentina
e-mail: chiapello@fcq.unc.edu.ar

C. T. Nuncira · M. D. Dib
Servicio de Dermatología, Hospital Pediátrico del Niño Jesús,
Córdoba, Argentina

clinical laboratories. In contrast, PCR fingerprinting has been previously demonstrated to be a simple and rapid method for detecting polymorphic DNA [10]. In particular, PCR employing the single repetitive oligonucleotides (GACA)₄, (GTG)₅ or M13 core sequence has been used for the identification of dermatophytes, although differences to discriminate *Trichophyton* species have been reported [11–13].

In contrast, epidemiological studies have been principally focused on detecting intraspecies differentiation of the genus *Trichophyton* [14–20], with few studies having investigated the molecular epidemiology of infections by strains of *Microsporium canis* [21–25]. In particular, RAPD-PCR has been applied to study an outbreak of *Tinea capitis* [22] and for detecting intraspecies variability in *M. canis* isolates [24, 25].

The objective of this study was to evaluate the capacity of the one-step PCR fingerprinting method (MSP-PCR) using the microsatellite primers (GACA)₄, M13 or (GTG)₅ to identify 82 isolates of dermatophytes from Argentinean patients with diverse clinical manifestations of dermatophytosis. Furthermore, we evaluated the intraspecies variability of 33 clinical isolates of *M. canis* by RAPD-PCR using the arbitrary decamer oligonucleotides OPK-20 and OPI-07 in order to study the epidemiological features of this species.

Methods

Cultures and fungal strains

Samples were collected at the Dermatology Service of the Hospital Pediátrico del Niño Jesús (Córdoba, Argentina) during the period 2007 to 2010 from skin, nail and hair of 894 individuals (children and adults) suspected of having cutaneous mycosis. Epidemiological data were recorded for all subjects, including age, sex, occupation, pet ownership, place of residence and clinical manifestations of the lesions. Individuals signed an informed consent and data were processed anonymously. Standard mycological identification was carried out according to the features of the fungal colonies and their morphologies observed under a microscope. A total of 82 dermatophyte isolates from clinical samples were included in the molecular studies, of which 73 strains were typed as *M. canis*, *Microsporium gypseum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Trichophyton tonsurans*. The remaining nine isolates could not be identified using the standard mycological identification (Table 1). In addition, six reference strains, including *M. canis* IP 2437.97, *M. gypseum* IP 137.62, *T. rubrum* IP 185.74, *T. mentagrophytes* IP 1182.79, *T. tonsurans* IP 1221.80 and *Trichophyton interdigitale* IP 2191.93 from the Pasteur Institute (Paris, France), were cultured for inclusion as controls in the PCR tests.

Extraction of genomic DNA for PCR

Total cellular DNA was extracted from the mycelium grown on Sabouraud agar slants after 10 days. Approximately 1 g of the mycelium was transferred to sterile mortars and liquid nitrogen was added. Then, the specimens were ground with a pestle until a fine powder was obtained, and the resulting powder was transferred to a 15-ml tube and mixed with 2 ml of lysis buffer (Tris-HCl 100 mM, EDTA 10 mM, ClNa 0.6 M, SDS 4 %) and 2 ml of phenol. The mycelium was vortexed for 15 min, and after centrifugation for 5 min at 6,000 rpm, 500 µl of the aqueous phase was transferred to a 1.5-ml Eppendorf tube. The DNA was successively extracted with phenol-chloroform (Sigma-Aldrich, St. Louis, MO, USA) and precipitated using 2 vols of sodium acetate solution and 1 vol of cold absolute ethanol. The pellet was washed with 0.5 ml of 70 % ethanol, air-dried and resuspended in buffer TE (10 mM TRIS-1 mM EDTA).

Microsatellite-primed PCR (MSP-PCR) and random amplification of polymorphic DNA (RAPD-PCR) methods

For the purpose of dermatophyte identification, MSP-PCR was performed using the two synthetic oligonucleotides (GACA)₄ and (GTG)₅, and the core sequence of phage M13 (GAGGGTGGCGTTCT) [26]. The reaction mixture was made with 25 ng of DNA in reaction buffer (10 mM Tris-HCl pH8.3 and 50 mM de MgCl₂) containing 200 µM of each deoxynucleoside triphosphate (dNTP) (Invitrogen), 160 ng of primer and 5 u of Platinum *Taq* DNA polymerase (Invitrogen). PCR amplification was carried out using a MultiGene™ Mini Personal Thermal Cycler (Labnet International, Edison, NJ, USA), performing 39 cycles of denaturation at 93 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. A tube with no template DNA was included as a negative control. The resulting PCR products were separated in 2 % agarose gels in TAE (Tris 4.8 %, 1.14 % acetic acid, 0.37 g% EDTA) at 50v for 75 min. The gel was stained in ethidium bromide or SYBR Safe (Invitrogen) diluted 1/10,000 and subsequently examined under UV light.

Analysis of the *M. canis* isolates by RAPD-PCR was carried out under the same conditions as those described above for MSP-PCR, using the primers OPI-07 (5-CAGCG ACAAG-3) and OPK-20 (5-GTGTGCGGAG-3) (Operon Technologies Inc., Alameda, CA, USA) [22].

The molecular patterns obtained in the agarose gels were analysed with Gel-Pro Analyzer software, which displays the profile of the band intensity on a graph of intensity versus molecular weight. PCRs were performed twice from the sample of DNA or using different extractions of DNA from the same fungus. In all cases, an identical band profile was obtained.

Table 1 Fungal strains analysed in this study and clinical information of patients from whom dermatophytes were isolated

Dermatophyte species	Total isolates	Type of dermatophytosis			Lesion ^b	
		<i>Tinea capitis</i>	<i>Tinea corporis</i>	<i>Tinea unguium</i>	Inflam.	Non-inflam.
<i>Microsporum canis</i>	46	34	12	–	4	42
<i>Trichophyton rubrum</i>	12	–	6	6	–	17
<i>Trichophyton mentagrophytes</i>	10	5	4	1	2	8
<i>Microsporum gypseum</i>	4	3	1	–	2	2
<i>Trichophyton tonsurans</i>	1	1	–	–	1	–
^a Unidentified dermatophytes	9	–	1	8	–	9

^a Identification was established by the typical micromorphology of colonies

^b Clinical presentation of lesions: inflammatory or non-inflammatory

Results

Identification of dermatophytes using MSP-PCR with (GACA)₄, M13 or (GTG)₅

The identification of certain species of dermatophytes using the microsatellite primers M13, (GTG)₅ and (GACA)₄ has been reported previously, but under different PCR conditions [12, 26]. In this study, PCR using (GACA)₄, M13 or (GTG)₅ as the single primer amplified all the studied strains, producing patterns of between one and seven bands, ranging from approximately 300 to 1,300 bp in length (Fig. 1). Furthermore, taking into account that *T. rubrum* or *T. mentagrophytes* can present similar phenotypic features and the same ecological niche as *T. interdigitale* [2], then the *T. interdigitale* IP 2191.93 reference strain from the Institut Pasteur was also included in the molecular analysis (Fig. 1). These results show that PCR using (GACA)₄ or (GTG)₅ produced specific band profiles of *T. rubrum*, *T. mentagrophytes* (clinical isolates) and *T. interdigitale* IP 2191.93. Therefore, all the clinical strains in our study were properly typified as *T. rubrum* or *T. mentagrophytes*, but *T. interdigitale* was not isolated from the patients.

PCR using (GACA)₄ produced superimposable profiles for all 46 clinical isolates and the reference strain of *M. canis*, revealing four bands of approximately 1,200, 1,000, 800 and 600 bp molecular weight. In a similar way, species-specific profiles were also observed for *M. gypseum*, *T. rubrum* and *T. interdigitale* IP 2191.93. However, DNA amplification with (GACA)₄ showed an identical, unique ~600-bp band for *T. mentagrophytes* and *T. tonsurans* (Fig. 1a and Online Resource 1).

PCR with M13 also produced specific profiles for *M. canis*, *M. gypseum* and *T. rubrum*. However, this primer was not useful for differentiating *T. mentagrophytes*, *T. tonsurans* and *T. interdigitale* IP 2191.93, demonstrating a similar band profile (Fig. 1b and Online Resource 2). The resulting band profiles for *M. canis* and *M. gypseum* with

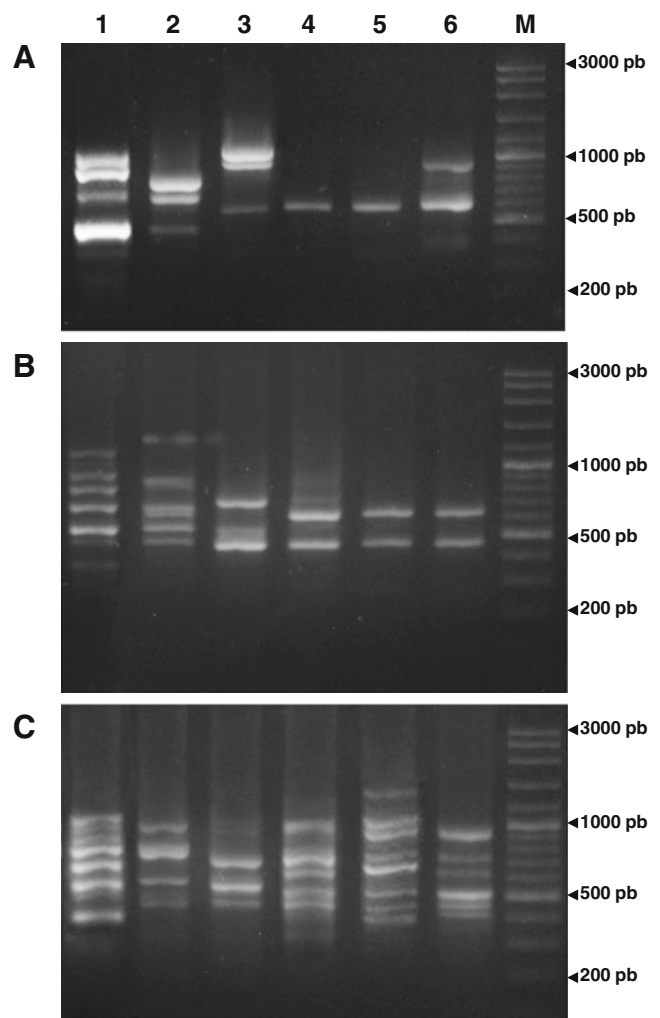


Fig. 1 DNA products from dermatophytes amplified by microsatellite-primed polymerase chain reaction (MSP-PCR) using the primers (a) (GACA)₄, (b) M13 and (c) (GTG)₅. Representative agarose gel electrophoresis for strains of *Microsporum canis* (lane 1), *Microsporum gypseum* (lane 2), *Trichophyton rubrum* (lane 3), *Trichophyton mentagrophytes* (lane 4), *Trichophyton tonsurans* (lane 5) and *Trichophyton interdigitale* IP 2191.93 (lane 6). M: molecular weight marker

this primer were more complex than those amplified by PCR using (GACA)₄ (Fig. 1b and Online Resource 2).

Finally, PCR with (GTG)₅ produced specific profiles for all the dermatophyte species analysed, revealing profiles with 3–7 bands of 300 to 1,100 bp molecular weight (Fig. 1c and Online Resource 3). These profiles of multiple bands were complex, with the molecular weights being close to each other, resulting in a less reproducible method than PCR using (GACA)₄. Furthermore, for each fungal species, PCR with (GTG)₅ showed identical patterns among the clinical isolates and the reference strain, except for *T. mentagrophytes*. In this latter case, all Argentinean *T. mentagrophytes* clinical isolates showed an identical pattern of five bands with ~1,000, 700, 600, 500 and 400 bp molecular weight. Although *T. mentagrophytes* IP 1182.79 also produced five bands, these were slightly different compared to the Argentinean clinical strains (~1,000, 700, 500, 450, 400 bp) (Online Resource 3).

In conclusion, based on the results presented here, a diagnostic algorithm is proposed taking into account the reproducibility and simplicity of the patterns obtained in the different PCRs. This involves performing a PCR with (GACA)₄ as a first step in the identification of the dermatophytes. Then, in the case that the PCR using (GACA)₄ shows a profile of *T. mentagrophytes* or *T. tonsurans*, PCR with (GTG)₅ should be applied next (Online Resource 4).

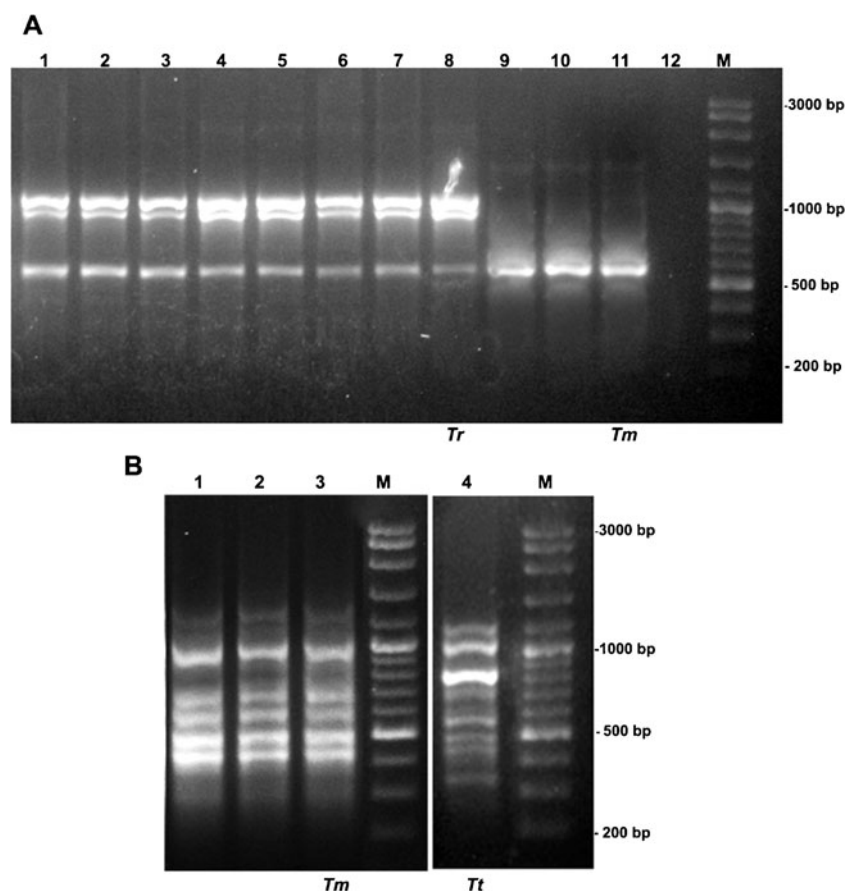
Application of MSP-PCR using (GACA)₄ and (GTG)₅ for the typification of previously unidentifiable dermatophytes by standard mycological techniques

The nine clinical isolates unidentifiable by standard mycological techniques (Table 1) were subjected to PCR using (GACA)₄ as the first step of molecular identification. The genetic patterns obtained are shown in Fig. 2a, revealing that seven isolates (lines 1–7) produced a band pattern corresponding to *T. rubrum* and two isolates (lines 9 and 10) matched the profile of *T. mentagrophytes* or *T. tonsurans*. Therefore, these latter two isolates were further subjected to PCR using (GTG)₅ and the results show that these two dermatophytes matched the profile of *T. mentagrophytes* (Fig. 2b).

Microsporum canis intragenic variability detection by RAPD-PCR using OPI-07 and OPK-20

We further investigated the intraspecies variability of 33 clinical isolates of *M. canis* by RAPD-PCR using OPK-20 and OPI-07. PCR using OPK-20 revealed five genotypes among the *M. canis* isolates, referred to as profiles 1, 2, 3, 4 and 5 (Online Resource 5). Profile 1 was the simplest and most frequent pattern, present in 26 isolates (70 %) with two

Fig. 2 MSP-PCR with (a) (GACA)₄ and (b) (GTG)₅ from dermatophytes unidentifiable by standard mycological techniques. **a** DNA profiles after PCR with (GACA)₄ from dermatophytes isolated from patients with onychomycosis (lanes 1–7 and lane 9) or *Tinea corporis* (lane 10); *T. rubrum* IP 185.74 (*Tr*, lane 8) and a local clinical strain of *T. mentagrophytes* (*Tm*, lane 11); negative control (lane 12), molecular weight marker (M). **b** DNA profiles after PCR with (GTG)₅ from dermatophytes that have shown a profile of *T. mentagrophytes* (*Tm*) or *T. tonsurans* (*Tt*) with PCR with (GACA)₄ in A (lanes 1 and 2), *T. mentagrophytes* (lane 3) and *T. tonsurans* IP 1221.80 (lane 4). M: molecular weight marker



bands of approximately 1,450 and 1,100 bp molecular weights. Profile 2 was produced by six isolates (16 %) and profiles 3, 4 and 5 were in the minority, corresponding to one or two strains each. No particular genotype was correlated with lesion localisation, clinical manifestations, geographic origin of the patients or pet ownership (data not shown).

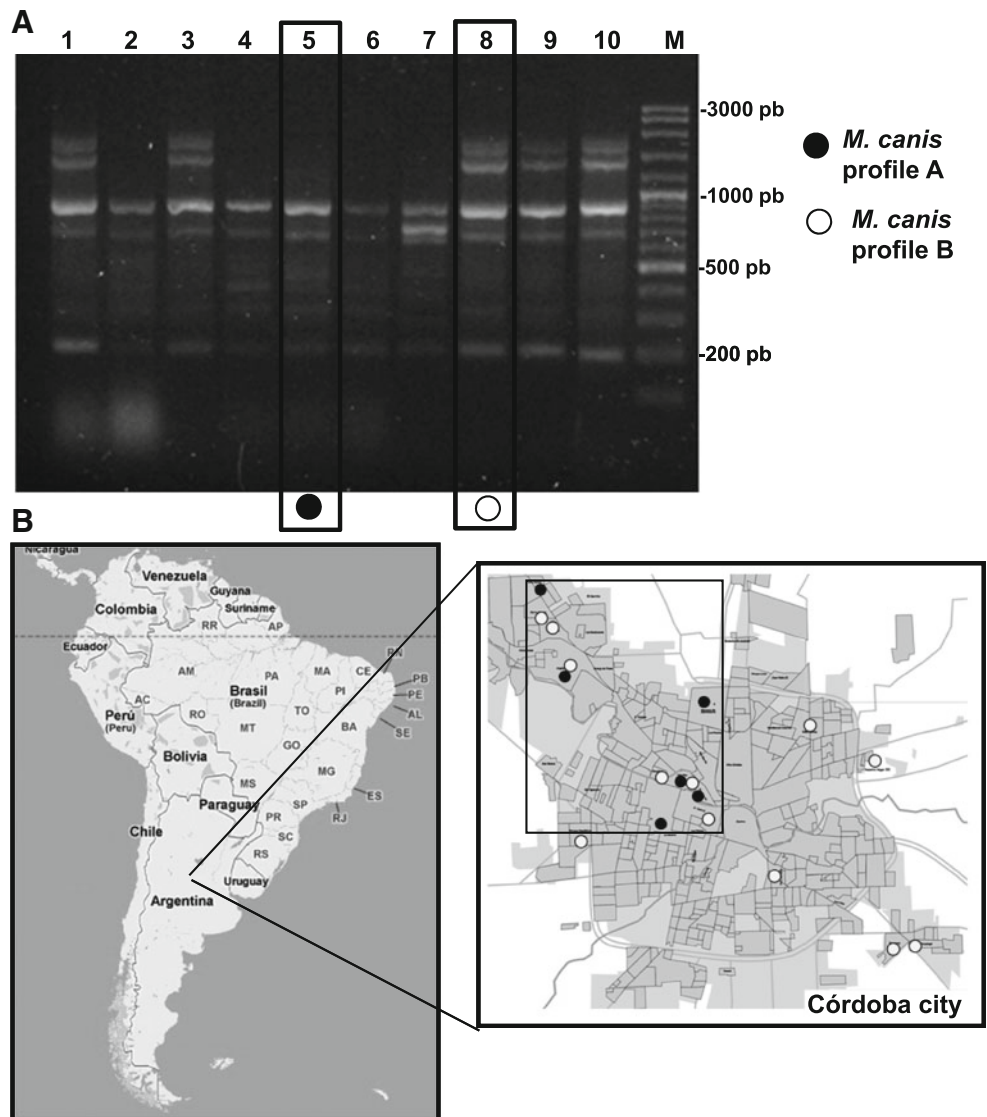
In contrast, PCR with OPI-07 produced two patterns of bands, referred to as profiles A and B. Profile A was produced by 19 isolates (57.5 %), showing two principal bands with a maximum intensity of approximately 950 and 700 bp, respectively. Profile B was found in 14 isolates (42.5 %), presenting five products with a maximum intensity of approximately 950, 700, 1,400, 1,750 and 1,850 bp molecular weights (Fig. 3a and Online Resource 6). In a similar way to that observed with OPK-20, these two genotypes were not associated to lesion localisation, clinical manifestations or pet ownership (data not shown). However, when we studied the origin of the patients from which the fungi were isolated,

we observed that strains with profile A showed a particular geographic restriction, since all cases were clustered in neighbourhoods belonging to the northwest area of the city (Fig. 3b).

Discussion

PCR fingerprinting has been previously demonstrated to be a fast and accurate method for detecting polymorphic DNA [10]. Here, we demonstrated that the application of PCR using the single microsatellite repetitive oligonucleotides (GACA)₄ and (GTG)₅ identified the most frequently found causative pathogen of dermatophytosis in less than 10 h from a pure colony of fungus. PCR using (GACA)₄ identified *T. rubrum*, *M. canis* and *M. gypseum*, which represent approximately 70 % of dermatophytes causing cutaneous mycosis in our country [27, 28] (M.F. Spesso, unpublished

Fig. 3 (a) DNA profiles obtained by random amplification of polymorphic DNA (RAPD-PCR) with the primer OPI-07 and (b) geographic distribution of *M. canis* clinical isolates. **a** Representative agarose gel electrophoresis after RAPD-PCR with OPI-07 from clinical isolates of *M. canis*, showing two band profiles, arbitrarily named A (black circle) and B (white circle). *M. canis* isolates from non-inflammatory *Tinea capitis* (lanes 1, 3, 5, 6, 7, 9 and 10), inflammatory *T. capitis* (lane 4) or *T. corporis* (lanes 2 and 8). M: molecular weight marker. **b** Geographic distribution showing the neighbourhoods of Córdoba city from which *M. canis* strains (profile A: black circles or profile B: white circles) were isolated



data). Similar results were also reported by Zhu et al. [11], demonstrating that (GACA)₄ was the most suitable primer to distinguish *T. rubrum*. However, (GACA)₄ produced different profiles among the *T. mentagrophytes* strains and it could not identify *T. interdigitale* [6, 12, 13, 26]. Under the conditions performed in this study, PCR with (GACA)₄ produced a specific pattern for *T. interdigitale* and was also suitable for distinguishing *T. rubrum* from *T. mentagrophytes*, which may present similar phenotypic features that make their identification difficult by culture. However, (GACA)₄ produced the same unique band for *T. mentagrophytes* and *T. tonsurans*. Similar results were reported by Roque et al. [26], who showed almost identical band profiles for these two species, although the band patterns were much more complex and had higher molecular weight bands. PCR using the primer M13 has been previously proposed as a simple, reliable and highly reproducible molecular tool to differentiate between strains of *Candida albicans* and *Candida dubliniensis* [29]. Nevertheless, in agreement with previous reports [11, 26], the results presented here showed that M13 did not improve the performance obtained with (GACA)₄.

Despite previous reports failing to identify *Trichophyton* species by PCR with (GTG)₅ [11, 26], under the conditions of this study, PCR with (GTG)₅ showed specific patterns for each dermatophyte species. However, the patterns were complex, and this primer suggests slight genetic differences occurring between *T. mentagrophytes* isolates from Argentina and France. Similar findings have also been reported for *Penicillium* spp. [30], and this has been attributed to (GTG)₅ recognising sequences with a high number of mutations.

Regarding dermatophyte identification, the results of this study demonstrate that the sequential application of PCR with (GACA)₄ and (GTG)₅ permitted the successful identification of nine clinical isolates of dermatophytes which had not been previously typified by classic mycological techniques.

In this work, intraspecies variability among the 33 clinical isolates of *M. canis* was detected using RAPD-PCR with the primers OPI-07 and OPK-20. Nevertheless, in contrast to that reported here and using the same method, Leibner-Ciszak et al. [25] were not able to detect genetic variations in 13 clinical isolates of *M. canis*. These discrepancies could have been due to different conditions in the PCR reaction or that the strains of *M. canis* used by these authors had no clonal diversity. Furthermore, in this study, RAPD-PCR did not detect any genotype variations associated with virulence or the animal origin of the strains. However, the finding of a particular geographical restriction of genotype A determined with OPK-20 suggests that our city may have a clonal diversity related to different ecological niches in certain geographical areas. Further studies with a greater number of isolates of the whole city should now be conducted in order to investigate this finding.

In conclusion, the results of this work demonstrate that the detection of intraspecies polymorphisms in isolates of *M. canis* by RAPD-PCR may be applied in future molecular epidemiological studies in order to identify endemic strains, the route of infection in an outbreak and the coexistence of different strains in a single infection.

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Conflict of interest The authors declare that they have no conflict of interest.

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