Specific Primers for Rapid Detection of *Microsporum audouinii* by PCR in Clinical Samples[▽]

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This report describes application of PCR fingerprinting to identify common species of dermatophytes using the microsatellite primers M13, (GACA)₄, and (GTG)₅. The initial PCR analysis rendered a specific DNA fragment for *Microsporum audouinii*, which was cloned and sequenced. Based on the sequencing data of this fragment, forward (MA_1F) and reverse (MA_1R) primers were designed and verified by PCR to establish their reliability in the diagnosis of *M. audouinii*. These primers produced a singular PCR band of 431 bp specific only to strains and isolates of *M. audouinii*, based on a global test of 182 strains/isolates belonging to 11 species of dermatophytes. These findings indicate these primers are reliable for diagnostic purposes, and we recommend their use in laboratory analysis.

Tinea (ringworm) is an infection of keratinized tissues (epidermis, hair, and nails) by a group of specialized fungi, the dermatophytes. Dermatophytes belong to three anamorphic genera, *Epidermophyton*, *Microsporum*, and *Trichophyton*, each of which includes several recognized species (28). Infections by dermatophytes result in symptoms that vary from mild to severe, depending on the immunological response of the host. Conventional procedures for routine identification of dermatophytes have mainly relied on microscopic examination of colony morphology, colony pigmentation, size and shape of macroconidia or microconidia, and colony growth rate under deficient conditions. Misidentification using conventional microbiological methods was one of the main reasons dermatophytes were among the first fungal groups studied using molecular genetic methods, such as the determination of nuclear DNA, G+C composition, and genomic DNA homology (6). These initial molecular approaches chiefly showed that dermatophytes, in general, were fundamentally closely related organisms.

Nonetheless, characterization by microscopic examination and in vitro culture of tinea are required for appropriate diagnosis and treatment, as well as for prevention of epidemic radiation of the infection. For example, in tinea capitis the identification of the species is important in the establishment of therapy. In general, infections by *Trichophyton* species need a longer period of treatment than those caused by *Microsporum* and *Microsporum canis* which only respond to terbinafine when higher doses are employed. Although in vitro culture is specific and sensitive, it has two significant drawbacks as a useful method for rapid diagnosis. These drawbacks are namely the long incubation period (2 or 3 weeks) required before diagnostic characteristics are developed in culture media and, secondly, the fact that many dermatophyte strains

often develop atypical characteristics (different colony morphologies, pleomorphism). The latter drawback can result in false-negative diagnosis in as many as 15% of cases where there is actual infection (24, 28). Moreover, misdiagnosis may lead to harmful or inadequate treatment.

Molecular biology techniques, such as arbitrarily primed PCR (16), random amplified polymorphic DNA analysis (21), restriction analysis of mitochondrial DNA (22), and microsatellite-primed PCR (MSP-PCR) (10–12, 25) are considered to be generally adequate for species identification of certain fungi. Such techniques have been used to differentiate 26 species of *Candida* and up to 17 species of dermatophytes, including intraspecific strains of some species. Nevertheless, species identification based on detection of numerous and complex PCR banding patterns generated by these techniques may be technically allusive and inconclusive. A practical and proven approach to overcome these technical deficiencies is to comparatively analyze individual DNA fragment profiles from the original PCR fingerprint in order to identify species- or strain-specific amplicons. Once such specific bands are identified, they can be sequenced and specific PCR primers/probes can be designed (9, 17, 18, 19).

In the present study, the application of PCR fingerprinting was performed using three sets of primers—M13, (GTG)₅, and (GACA)₄—for the purpose of species identification and strain typing of common dermatophytes and related fungi. During analysis of the fingerprints of 11 species of dermatophytes, a specific DNA fragment was detected to be exclusive to only *Microsporum audouinii* and specific for all strains examined. Based on the DNA sequence of this amplicon, species-specific PCR primers were designed and tested for rapid and efficient PCR-based detection and to evaluate the diagnostic value of this method.

MATERIALS AND METHODS

Cultures. During 2002 and 2003, samples were collected from skin, nail, and hair from patients suspected of having ringworm as putatively diagnosed in various dermatology clinics in the Lisbon area. A small sample from each of the specimens was suspended in a drop of 20% (wt/vol) KOH and examined under

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TABLE 1. 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 samples) from patients by:						
		Skin	Nail	Hair	ND ^a	Sex			Age (yr)			
						Male	Female	ND	≤18	>18 and ≤50	>50	ND
<i>T. ajelloi</i>	1	100 (1)	0	0	0	100 (1)	0	0	0	0	100 (1)	0
<i>T. rubrum</i>	36	77.8 (28)	19.4 (7)	0	2.7 (1)	69.4 (25)	30.6 (11)	0	2.8 (1)	44.4 (16)	38.9 (14)	13.9 (5)
<i>T. mentagrophytes</i>	12	58.3 (7)	33.3 (4)	8.3 (1)	0	41.7 (5)	58.3 (7)	0	16.7 (2)	66.7 (8)	8.3 (1)	8.3 (1)
<i>T. mentagrophytes</i> var. <i>granulare</i>	10	50 (5)	50 (5)	0	0	60 (6)	40 (4)	0	50 (5)	10 (1)	40 (4)	0
<i>T. interdigitale</i>	4	75 (3)	25 (1)	0	0	25 (1)	75 (3)	0	0	50 (2)	25 (1)	25 (1)
<i>T. megninii</i>	2	100 (2)	0	0	0	50 (1)	50 (1)	0	0	50 (1)	50 (1)	0
<i>T. tonsurans</i>	11	27.3 (3)	27.3 (3)	0	45.4 (5)	50 (3)	50 (3)	45.4 (6)	54.5 (6)	0	0	41.7 (5)
<i>T. soudanense</i>	12	8.3 (1)	0	91.7 (11)	0	41.7 (5)	58.3 (7)	0	75 (9)	0	8.3 (1)	16.7 (2)
<i>M. canis</i>	26	46.4 (13)	0	42.9 (12)	3.8 (1)	28 (7)	64 (16)	11.5 (3)	53.8 (14)	23.1 (6)	4.3 (1)	19.2 (5)
<i>M. audouinii</i>	27	7.4 (2)	0	92.6 (25)	0	66.7 (18)	33.3 (9)	0	92.6 (25)	3.7 (1)	0	3.7 (1)
<i>M. gypseum</i>	3	100 (3)	0	0	0	66.7 (2)	33.3 (1)	0	66.7 (2)	33.3 (1)	0	0
<i>E. floccosum</i>	3	100 (3)	0	0	0	100 (3)	0	0	0	0	100 (3)	0

^a ND, data not available.

a microscope. The remaining portion of each specimen was inoculated onto Sabouraud dextrose agar (Difco) and cultured at 25°C for up to 4 weeks. Identification of dermatophyte isolates obtained from these cultures was achieved by conventional microscopic techniques (28).

A total of 172 dermatophyte isolates representing three *Microsporum* species, seven *Trichophyton* species, and *Epidermophyton floccosum* were included in this study (Table 1). Ten reference strains, including *Microsporum canis*, *M. audouinii*, *Trichophyton interdigitale*, *Trichophyton mentagrophytes* var. *erinacei*, *T. mentagrophytes* var. *goetzii*, *Trichophyton ajelloi*, *Trichophyton rubrum*, *Arthroderma vanbreuseghemii* (mating types + and -), and *Epidermophyton floccosum* (Table 2) were cultured for inclusion as positive controls in PCR tests.

At least two clinical isolates of other common filamentous fungi, such as *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus flavus*, and one clinical isolate of *Fonsecaea pedrosoi*, as well as clinical isolates of *Candida albicans* (including strain ATCC 22019), *Candida krusei*, *Candida parapsilosis* (including strain ATCC 90028), *Candida glabrata*, *Candida tropicalis*, *Candida guilliermondii*, and *Cryptococcus neoformans* (CBS 132^T) were also cultured for PCR tests.

Extraction of genomic DNA for PCR. A rapid DNA extraction technique for filamentous fungi (26) was used with some slight modifications. Briefly, a portion of mycelium from cultures on Sabouraud dextrose broth was added to a 1.5-ml tube containing 500 µl of lysis buffer (50 mM Tris liter⁻¹, pH 8.0, 50 mM EDTA liter⁻¹, pH 8.0, 250 mM NaCl liter⁻¹, 0.3% [wt/vol] sodium dodecyl sulfate [SDS], pH 8.0), and 500 mg of acid-washed 0.4- to 0.6-mm-diameter glass beads (Sigma, St. Louis, MO).

Mycelia were lysed by continuous vortexing for 15 min on a Labincor BV-L46 vortex (Labincor, Breda, The Netherlands) at the highest intensity setting. Vortexed tubes were incubated at 65°C for 1 h and then revortexed for additional 10 min. Lysed suspensions were centrifuged at 13,000 × g for 15 min at 4°C, and supernatants were transferred to 1.5-ml tubes. Proteinase K (Gibco, BRL, Eggenstein, Germany) was added to a final concentration of 500 µl ml⁻¹. Tubes were incubated at 60°C for 60 min (13) and centrifuged at 13,000 × g for 15 min, and the supernatant was transferred to new 1.5-ml tubes and diluted 1:750 in 10 mM Tris-HCl (pH 8.0).

Human DNA to be used in PCR tests was extracted from blood, using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions for 300 µl of whole blood.

Microsatellite-primed PCR. The core sequence of phage M13 (GAGGGTG GCGTTCT) was used as a single primer in minisatellite-primed PCR experiments (20), and two synthetic oligonucleotides, (GACA)₄ and (GTG)₅, were tested with microsatellite-primed PCR experiments (20) (MWG-Biotech AG, Ebersberg, Germany). MSP-PCR was performed in a 25-µl reaction mixture containing 10 to 15 ng of genomic DNA (5 µl from Tris-HCl dilution), 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 9.0), 0.01% Tween 20, 2 mM MgCl₂, 250 µM each deoxynucleoside triphosphate (dNTP), 1 U of *Taq* DNA polymerase (Bio-line, London, United Kingdom), and 0.8 µM of PCR primers as previously described (23). PCR amplification was performed using a *Tpersonal* Combi PCR system (Biometra, Göttingen, Germany) with the following thermocycling pro-

gram: 1 cycle at 95°C for 5 min; 40 cycles at 93°C for 40 s, 50°C for 60 s, and 72°C for 60 s; and a final cycle at 72°C for 6 min. A tube having no DNA was included as a template control. After completion of amplification, 10 µl of the reaction mixture was added to 3 µl of 10× DNA sample buffer containing bromophenol and glycerol. DNA fragments were separated by electrophoresis in 1% (wt/vol) agarose gels in 0.5× Tris-borate-EDTA buffer at 50 V for 5 h. The gel was stained in ethidium bromide for 10 min and subsequently examined under UV light. Sizes of amplified DNA fragments were estimated using a GeneRuler 100-bp DNA Ladder Plus (MBI, Fermentas, Germany).

DNA cloning, sequencing, and primer design. Fragments generated by MSP-PCR were excised from gels and purified using Gel Extraction Jetquick (Genomed, Oeynhausen, Germany). Purified fragments were then cloned into plasmid vectors (pBluescript II KS [+]) (Stratagene, Integrated Sciences, Melbourne, Australia), according to the manufacturer's instructions. Cloned DNA fragments used for sequencing were amplified by PCR using 50 µM of T3 and T7 primers (Invitrogen, Carlsbad, Calif.), and purified using the Jetquick/PCR purification kit (Genomed, Oeynhausen, Germany), according to the manufacturer's instructions. Sequencing was performed in an automatic Beckman Coulter 2000 XL sequencer using the T3 and T7 primers. PCR primers specific to the *M. audouinii* fragment were designed with the aid of the online computer program Primer Design Assistant (PDA) (5). Selected primers were then artificially synthesized (MWG-Biotech AG, Ebersberg, Germany).

***M. audouinii*-specific PCR.** PCR using *M. audouinii*-specific primers was performed as described for MSP-PCR, but with 0.4 µM of each *M. audouinii* species-specific primer and the following thermal cycle program: one cycle at 95°C for 3 min and 35 cycles at 93°C for 30 s, 62°C for 30 s, and 72°C for 30 s. A tube with no template DNA was included as a negative control. The resultant PCR products were examined in the same fashion as for MSP-PCR.

Multiplex PCR for assessment of reliability of identification. *M. audouinii*-specific primers were used in a PCR analysis under the same conditions described above, but with 0.2 µM universal primers for fungi TR1 (5'-GTTTCT AGGACCGCCGTA-3') and TR2 (5'-CTCAAACTTCCATCGACT-3') (2) added to the reaction mixture. The PCR thermocycle program was the same as that used for the *M. audouinii* species-specific PCR. A blank tube having no template served as a negative control.

RESULTS

Clinical profile. Clinical data concerning patients from which dermatophytes were identified are summarized in Table 1. *T. rubrum* was the most prevalent species identified, collected exclusively from skin and nail samples, mainly from male patients above 18 years in age. *M. audouinii* was the second most prevalent species detected, obtained almost exclusively from hair samples from children at a ratio of 2:1 male

TABLE 2. Dermatophytes, other filamentous fungi, and yeast strains studied

Organism	Source ^a	No. tested
Dermatophytes		
<i>Trichophyton ajelloi</i> ^T	CECT 2991	1
	Clinical (LM0409dt)	1
<i>T. mentagrophytes</i> var. <i>erinacei</i>	CECT 2956	1
<i>T. mentagrophytes</i> var. <i>goetzii</i>	CECT 2957	1
<i>T. interdigitale</i>	CECT 2958	1
	Clinical (LM9302dt)	4
<i>T. mentagrophytes</i> var. <i>granulare</i>	Clinical (LM9304t)	10
	Clinical, dog (LM9304dt)	2
<i>T. mentagrophytes</i>	Clinical (LM9805dt)	12
<i>T. megninii</i>	Clinical (LM9301dt)	2
<i>T. rubrum</i>	CECT 2794	1
	Clinical (LM9107dt)	36
<i>T. soudanense</i>	Clinical (LM9503dt)	12
<i>T. tonsurans</i>	Clinical (LM9203dt)	11
<i>Microsporum audouinii</i>	DSMZ 10649	1
	Clinical (LM9104dm)	27
<i>M. canis</i>	DSMZ 10708	1
	Clinical (LM9110dm)	26
<i>M. gypseum</i>	Clinical (LM9912dm)	3
<i>Epidermophyton floccosum</i>	CECT 2796	1
	Clinical (LM9202dm)	3
<i>Arthroderma vanbreuseghemii</i> mating type +	CECT 2900	1
<i>Arthroderma vanbreuseghemii</i> mating type –		
Unidentified	Clinical	23
Subtotal		182
Other molds		
<i>Aspergillus flavus</i>	Clinical (LM9502fa)	2
<i>A. fumigatus</i>	Clinical (LM9706fa)	3
<i>A. niger</i>	Clinical (LM9304fa)	2
<i>A. terreus</i>	Clinical (LM0408fa)	2
<i>Fonseca pedrosoi</i>	Clinical (LM0402ff)	1
Subtotal		10
Yeasts		
<i>Candida albicans</i>	ATCC 90028	1
	Clinical (LM9104lc)	5
<i>C. parapsilosis</i>	ATCC 22019	1
	Clinical (LM9104lca)	4
<i>C. glabrata</i>	Clinical (LM9412lc)	3
<i>C. krusei</i>	Clinical (LM9105lc)	1
<i>C. tropicalis</i>	Clinical (LM9105lca)	1
<i>C. guilliermondii</i>	Clinical (LM9302lc)	1
Subtotal		17
Total		209

^a CECT, Colección Española de Cepas Tipo, València, Spain; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; ATCC, American Type Culture Collection, Manassas, Va. The numbers in parentheses are the species designation at the Mycology Laboratory in the Institute of Hygiene and Tropical Medicine.

to female. The third most prevalent species was *M. canis*, isolated mainly from skin and hair samples of children at a ratio of 1:2 male to female. *T. mentagrophytes* was isolated mostly from skin and nails, from all age groups ≤50 years,

equally from both genders. *T. mentagrophytes* var. *granulare* was identified exclusively from skin and nail samples, mostly in the young (≤18 years) and oldest (>50 years) patients, equally in both genders. *T. tonsurans* was isolated from skin and nail samples from both male and female patients ≤18 years of age. These clinical profiles were similar for those of patients from whom *Trichophyton soudanense* was isolated, except the isolation occurred chiefly from hair.

Microsatellite-primed PCR. 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 (11, 12). Figure 1 shows the electrophoretic band patterns obtained with primers M13, (GTG)₅, and (GACA)₄ under our PCR conditions. All sets of primers generated distinct PCR band profiles of each dermatophyte, with bands varying in intensities and molecular sizes. The PCR fingerprints showed DNA fragments that ranged from 0.2 to 4.9 kb in length for all species. Figure 1 also shows that all 11 species studied could be differentiated with the primer sets used, with the following exceptions: primer (GTG)₅ failed to distinguish *T. rubrum*, *T. soudanense*, and *T. megninii* (Fig. 1B), whereas *T. soudanense* and *T. megninii* were indistinguishable with primer (GACA)₄ (Fig. 1C). On the other hand, *T. rubrum* and *T. soudanense* presented similar DNA fragment patterns with primer M13 (Fig. 1A). None of the primers was able to distinguish the mating types of *A. vanbreuseghemii* (Fig. 1), whose patterns were quite similar to those of *T. mentagrophytes*, *T. mentagrophytes* var. *goetzii*, *T. interdigitale*, and *T. mentagrophytes* var. *granulare* (Fig. 1). All primers distinguished *T. mentagrophytes* var. *erinacei* from the other varieties of *T. mentagrophytes* of this study. Nevertheless, the remaining varieties could not be differentiated by any of the three primers used.

Only one isolate of *M. audouinii*, misidentified by visible morphological methods, generated a similar DNA banding profile to that of *T. interdigitale* (CECT 2958) for all primers tested (data not shown). Of the isolates that could not be identified by conventional methods, four produced similar band patterns to those of *M. audouinii* with all primers and were thus categorized as being *M. audouinii* (Fig. 1A). The remaining isolates of *M. audouinii* produced similar patterns with the different primers, with the exception of two isolates identified as *M. audouinii* with primer (GACA)₄ and as *M. canis* with primers M13 and (GTG)₅ (data not shown).

Analysis of DNA sequences of unique bands for species-specific identification. The M13 primer produced a clearer distinction between *M. canis* and *M. audouinii* than the other primers used. A prominent species-specific fragment, of approximately 0.9 kb, generated from the genomic DNA of *M. audouinii* (reference strain DSMZ 10649) with the M13 primer (Fig. 1A), was excised from the gel, purified, cloned, and sequenced. The sequence product of the cloned amplicon was 919 bp in length. From this sequence, a forward primer, MA1_F (5'-CGAAGCGAGCCTCTACGGCAATCCAAAGCAG-3'), and a reverse primer, MA1_R (5'-CGAAGCATCTTGCAGGACTCC-3'), were designed according to protocols outlined in Materials and Methods. These primers generated an amplicon of 431 bp. The primers were tested under several PCR conditions and with the genomic DNA of all the strains/isolates used in this study.

FIG. 1. DNA products from dermatophytes amplified by microsatellite-primed PCR using primers M13 (A), (GTG)₅ (B), and (GACA)₄ (C). (A) Lanes 1 and 22 contain molecular size markers (in bp) at the left and right margins, respectively. Lane 2, DNA products generated from *M. audouinii* DSMZ 10649; lanes 3 and 4, *M. audouinii* LM9104dm; lanes 5 and 6, *M. audouinii* strain unidentified; lane 7, *M. canis* DSMZ 10708; lane 8, *E. floccosum* CECT 2796; lane 9, *T. ajelloi* CECT 2991^T; lane 10, *T. mentagrophytes* var. *erinaceii* CECT 2956; lane 11, *T. mentagrophytes* var. *goetzii* CECT 2957; lane 12, *T. interdigitale* CECT 2958; lane 13, *T. mentagrophytes* var. *granulare* LM9304t; lane 14, *T. mentagrophytes* LM9805dt; lane 15, *A. vanbreuseghemii* CECT 2900 (mating type +); lane 16, *A. vanbreuseghemii* CECT 2899 (mating type -); lane 17, *T. rubrum* CECT 2794; lane 18, *T. tonsurans* LM9203dt; lane 19, *T. soudanense* LM9503dt; lane 20, *T. megninii* LM9301dt; lane 21, *M. gypseum* LM9912dm. The arrow points to the specific DNA fragment of *M. audouinii*. (B and C) Lanes 1 and 18 contain molecular size markers (in bp) at the left and right margins, respectively. Lane 2, DNA products generated from *M. audouinii* DSMZ 10649; lane 3, *M. canis* DSMZ 10708; lane 4, *E. floccosum* CECT 2796; lane 5, *T. ajelloi* CECT 2991^T; lane 6, *T. mentagrophytes* var. *erinaceii* CECT 2956; lane 7, *T. mentagrophytes* var. *goetzii* CECT 2957; lane 8, *T. interdigitale* CECT 2958; lane 9, *T. mentagrophytes* var. *granulare* LM9304t; lane 10, *T. mentagrophytes* LM9805dt; lane 11, *A.*

Identification reliability assessment of “MA1” primers. The utility of the MA1_F and MA1_R primers as a tool for identifying *M. audouinii* (4) was compared to that of the microsatellite primers M13, (GTG)₅, and (GACA)₄. All strains used in this study were tested with these primers in a Multiplex PCR with universal primers. As shown in Fig. 2, the expected band of 431 bp, using the MA1 primers, was generated only with strains of *M. audouinii*, whereas a band of 600 bp was generated by primers TR1 and TR2 for all fungal species, including *M. audouinii*. In some cases, the 600-bp fragment was weak or absent for the strain/isolates of *M. audouinii*. The four isolates that could not be identified by conventional methods, but were identified as *M. audouinii* by MSP-PCR fingerprinting, produced the 431-bp fragment when tested with the MA1 primers.

In order to assess the identification potential for PCR fingerprinting and the *M. audouinii*-specific PCR, parameters were set up to evaluate the reliability and accuracy for identification and diagnostic purposes (Table 3). This evaluation was performed with the consideration that ordinarily identification is made by morphological features. We found that all of the primers tested had values showing a high degree of detection sensitivity and specificity (above 89% and 98%, respectively) (see Discussion). Positive and negative predictive values were also congruent with there being a high identification potential of the PCR techniques developed in this study.

DISCUSSION

Infections of humans by dermatophytes have historically been considered to be of little particular medical importance, in view that such infections were generally mild and not life-threatening. Moreover, many of the more significant etiologic agents were restricted to specific geographic areas and not involved in widespread epidemics. However, demographic changes over the prior two decades have resulted in widespread infections caused by a variety of species of dermatophytes. Nowadays, cutaneous infections caused by dermatophytic fungi occur in every age group worldwide. The millions of individuals who are infected by these pathogens contribute to health care costs in the tens of millions of dollars on an annual basis (8), especially for treatment when diagnosis is not quickly established.

Over the past few years, the number of cases of tinea capitis due to anthropophyllic fungi has significantly increased, particularly in some European city communities (1, 3, 7). In Portugal, recent epidemiological data on dermatophyte infections are not available. In a study of isolates from a clinical laboratory attending to patients from the Lisbon area, *T. rubrum* was the most frequently isolated agent of dermatophytosis (28.5%), with *M. audouinii* (22.6%) and *T. soudanense* (14.3%) being the dermatophytes isolated the second and third most often, respectively. Tinea capitis was the most diagnosed dermatophytosis, followed by tinea pedis. This pattern in the Por-

vanbreuseghemii CECT 2900 (mating type +); lane 12, *A. vanbreuseghemii* CECT 2899 (mating type -); lane 13, *T. rubrum* CECT 2794; lane 14, *T. tonsurans* LM9203dt; lane 15, *T. soudanense* LM9503dt; lane 16, *T. megninii* LM9301dt; lane 17, *M. gypseum* LM9912dm.

FIG. 2. DNA products amplified in PCR using *M. audouinii*-specific primers MA1_F and MA1_R and universal primers TR1 and TR2. Lanes 1, 19, 20, and 34 contain DNA molecular size marker 100-bp DNA Ladder Plus (MBI, Fermentas, Germany), with molecular sizes (in bp) shown on the left and right; lanes 18 and 33 are negative controls. Lane 2, DNA products generated from *M. audouinii* DSMZ 10649; lane 3, *M. canis* DSMZ 10708; lane 4, *E. floccosum* CECT 2796; lane 5, *T. ajelloi* CECT 2991^T; lane 6, *T. mentagrophytes* var. *erinacei* CECT 2956; lane 7, *T. mentagrophytes* var. *goetzii* CECT 2957; lane 8, *T. interdigitale* CECT 2958; lane 9, *T. mentagrophytes* var. *granulare* LM9304t; lane 10, *T. mentagrophytes* LM9805dt; lane 11, *A. vanbreuseghemii* CECT 2900 (mating type +); lane 12, *A. vanbreuseghemii* CECT 2899 (mating type –); lane 13, *T. rubrum* CECT 2794; lane 14, *T. tonsurans* LM9203dt; lane 15, *T. soudanense* LM9503dt; lane 16, *T. megninii* LM9301dt; lane 17, *M. gypseum* LM9912dm; lane 18, *E. floccosum*; lanes 21 to 28, *M. audouinii*; lanes 29 to 32, *M. audouinii* (unidentified).

tuguese clinic is distinct from the one observed throughout other countries in western Europe but is similar to that found in some African countries (P. Viegas, A. Pinto, A. Santos, M. Pinheiro, and R. Vieira, Reun. Centro Soc. Port. Dermat. Vener., abstr.72, 2003). In our laboratory, skin-related dermatophytes were the most frequently identified organisms, followed by tinea capitis. The most isolated agent was *T. rubrum*, followed by *M. audouinii* and *M. canis* (Table 1).

In Portugal, there are recent data suggesting *M. audouinii* and *T. soudanense* as the dermatophytes isolated most frequently from tinea capitis. These fungi, prevalent in African countries, are now infecting European children and are increasingly responsible for outbreaks in European schools (27). Our records are congruent with these data. Still, *M. canis* is a major tinea capitis etiologic agent. Its widespread occurrence and capability of infecting distinct tissues account for the high number of hair infections detected (Table 1).

In this study, differentiation of species involved in tinea capitis was possible by MSP-PCR using three different primers. As previously described, it was possible to discriminate among all the species with primers (GACA)₄ (11) and M13 and (GTG)₅ (12). Variability among varieties of *T. mentagrophytes*

was observed for *T. mentagrophytes* var. *erinacei* (CECT 2956) for all three primers. Slight differences were observed among the remaining varieties of *T. mentagrophytes* with these primers as well. Similarity in fingerprinting patterns between *A. vanbreuseghemii* (both mating types) and varieties of *T. mentagrophytes* (with the exception of *T. mentagrophytes* var. *erinacei*) reflects the close phylogenetic relationship among them (14). The failure of primers (GTG)₅ and (GACA)₄ to discriminate *T. soudanense* and *T. megninii* is evidence of their close phylogenetic proximity. In fact, in a previous report (15), *T. soudanense* was reclassified as *Trichophyton violaceum* and *T. megninii* was considered an ambiguous taxonomic classification. These three species belong to the *T. rubrum* complex, as shown by the high degree of similarity in their DNA fragment patterns in our study.

Application of MSP-PCR has enabled rapid identification of several dermatophyte species/varieties (10–12). Nevertheless, development of specific primers for different species would improve specificity of PCR-based tests and eliminate problems of ambiguities in band pattern analysis. Development of a *T. rubrum*-specific probe (9) and, more recently, a specific *M.*

TABLE 3. Reliability of PCR identification of *M. audouinii* compared with the standard method of identification based on morphological features

Primer	No. (%) of strains with result ^a :							
	True positive	False positive	True negative	False negative	Sensitivity	Specificity	PPV	NPV
MA1	27 (12.9)	0 (0.0)	181 (86.6)	1 (0.5)	96.4	100	100	99.5
M13	25 (12.0)	2 (1.0)	179 (85.6)	3 (1.4)	89.3	98.9	92.6	98.4
(GTG) ₅	25 (12.0)	3 (1.4)	178 (85.2)	3 (1.4)	89.3	98.3	89.3	98.3
(GACA) ₄	27 (12.9)	2 (1.0)	179 (85.6)	1 (0.5)	96.4	98.9	93.1	99.4

^a Values in parentheses refer to percentages of the total number of strains (*n* = 209). PPV, positive predictive value; NPV, negative predictive value.

canis primer set (18) has demonstrated the diagnostic importance of such primer-specific approaches.

Difficulty in identifying *M. audouinii* by macro- and microscopic features, especially in distinguishing it from *M. canis*, and time-consuming laboratory diagnosis led us to design a specific primer for identification of *M. audouinii*. To ensure that no false-negative results were obtained from the PCRs using our MA1 primers, a universal primer set generating an amplicon of 600 bp was added to serve as a positive control of the PCR. This mix of primer sets produced two fragments (of 600 bp and 431 bp; Fig. 2) in the strains/isolates of *M. audouinii* and just one fragment of 600 bp in the non-*M. audouinii* strains/isolates (Fig. 2).

The reliability of primers MA1_F and MA1_R as a diagnostic tool showed the MA1 primers had high specificity, sensibility, PPV, and PVN rates (all values near 100%) for the strains/isolates tested (Table 3). It is worth mentioning that two of the false-positive tests obtained with primers M13 and (GTG)₅ were attributed to two isolates of *M. audouinii* that were identified as *M. canis* by M13 and (GTG)₅ and as *M. audouinii* by (GACA)₄ and MA1. Considering these findings, we conclude the two isolates are *M. audouinii*. Also, the one false-negative resulting in all primer tests was attributed to one isolate identified as *M. audouinii* by morphological features. However, the MSP-PCR primers identified this isolate as *T. interdigitale*. Moreover, this isolate also did not produce the 431-bp band expected for *M. audouinii* with MA1 primers. This shows that MA1 primers had 100% accuracy in the study sample.

In conclusion, the results of our study strongly support use of the MA1 *M. audouinii*-specific-primers for the identification of *M. audouinii* in the diagnosis of tinea capitis. In the future, PCR-based identification, as outlined in this study, can be applied directly to skin and hair samples. This has the advantage relative to conventional culture methods of making identifications in a shorter time-frame (1 to 4 days instead of 2 to 3 weeks) and eliminating problems of culture failure and contamination. The PCR approach does rely, however, on efficient extraction of nucleic acids that avoids possible degradation of DNA, which could lead to false-negative results. In our laboratory, we have already started to use this technique as a diagnostic tool in parallel with the conventional methods. So far, results have been 100% compatible. Moreover, detection of dermatophytes by the PCR technique instead of culture will allow rapid diagnosis of the species that will lead to a better management of infections caused by these fungi.

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REFERENCES

- Aly, R., R. Hay, A. Del Palacio, and R. Galimberti. 2000. Epidemiology of tinea capitis. *Med. Mycol.* 38(Suppl. 1):183-188.
- Bock, M., M. Maiwald, R. Kappe, P. Nickel, and H. Naher. 1994. Polymerase chain reaction-based detection of dermatophyte DNA with a fungus-specific primer system. *Mycoses* 37:79-84.
- Brilhante, R., G. Paixao, L. Salvino, M. Diogenes, S. Bandeira, M. Rocha, J. dos Santos, and J. Sidrim. 2000. Epidemiology and ecology of dermatophytoses in the City of Fortaleza: *Trichophyton tonsurans* as important emerging pathogen of tinea capitis. *Rev. Soc. Bras. Med. Trop.* 33:417-425.
- Campbell, M. J., and D. Machin. 1990. Medical statistics: a commonsense approach. John Wiley & Sons, Ltd., Chichester, United Kingdom.
- Chen, S. H., C. Y. Lin, C. S. Cho, C. Z. Lo, and C. A. Hsiung. 2003. Primer Design Assistant (PDA): a web-based primer design tool. *Nucleic Acids Res.* 31:3751-3754.
- Davison, F., and D. Mackenzie. 1984. DNA homology studies in the taxonomy of dermatophytes. *Sabouraudia* 22:117-123.
- Devliotou-Panagiotidou, D., T. Koussidou-Eremondi, G. Chaidemenos, M. Theodoridou, and A. Minas. 2001. Tinea capitis in adults during 1981-95 in northern Greece. *Mycoses* 44:398-400.
- Drake, L., S. Dinehart, E. Farmer, R. Goltz, G. Graham, M. Hardinsky, C. Lewis, D. Pariser, J. Skouge, S. Webster, D. Whitaker, B. Butler, B. Lowery, B. Elewski, M. Elgart, P. Jacobs, J. Leshner, R. Scher, et al. 1996. Guidelines of care for superficial mycotic infections of the skin: tinea corporis, tinea cruris, tinea faciei, tinea manuum, and tinea pedis. *J. Am. Acad. Dermatol.* 34:282-286.
- El Fari, M., H. Tietz, W. Presber, W. Sterry, and Y. Graser. 1999. Development of an oligonucleotide probe specific for *Trichophyton rubrum*. *Br. J. Dermatol.* 141:240-245.
- Faggi, E., G. Pini, and E. Campisi. 2002. PCR fingerprinting for identification of common species of dermatophytes. *J. Clin. Microbiol.* 40:4804-4805. (Letter.)
- Faggi, E., G. Pini, E. Campisi, C. Bertellini, E. Difonzo, and F. Mancianti. 2001. Application of PCR to distinguish common species of dermatophytes. *J. Clin. Microbiol.* 39:3382-3385.
- Graser, Y., M. el Fari, W. Presber, W. Sterry, and H. Tietz. 1998. Identification of common dermatophytes (*Trichophyton*, *Microsporum*, *Epidermophyton*) using polymerase chain reactions. *Br. J. Dermatol.* 138:576-582.
- Graser, Y., A. Kuijpers, M. El Fari, W. Presber, and G. de Hoog. 2000. Molecular and conventional taxonomy of the *Microsporum canis* complex. *Med. Mycol.* 38:143-153.
- Graser, Y., A. Kuijpers, W. Presber, and G. De Hoog. 1999. Molecular taxonomy of *Trichophyton mentagrophytes* and *T. tonsurans*. *Med. Mycol.* 37:315-330.
- Gräser, Y., A. F. A. Kuijpers, W. Presber, and G. S. de Hoog. 2000. Molecular taxonomy of the *Trichophyton rubrum* complex. *J. Clin. Microbiol.* 38:3329-3336.
- Liu, D., S. Coloe, J. Pedersen, and R. Baird. 1996. Use of arbitrarily primed polymerase chain reaction to differentiate *Trichophyton* dermatophytes. *FEMS Microbiol. Lett.* 136:147-150.
- Liu, D., L. Pearce, G. Lilley, S. Coloe, R. Baird, and J. Pedersen. 2002. PCR identification of dermatophyte fungi *Trichophyton rubrum*, *T. soudanense* and *T. gourvilii*. *J. Med. Microbiol.* 51:117-122.
- Liu, D., L. Pearce, G. Lilley, S. Coloe, R. Baird, and J. Pedersen. 2001. A specific PCR assay for the dermatophyte fungus *Microsporum canis*. *Med. Mycol.* 39:215-219.
- Martins, M. L. M. 2001. Polimorfismos fenéticos e diagnóstico molecular de *Cryptococcus neoformans*. Ph.D. thesis. Universidade Nova de Lisboa, Lisbon, Portugal.
- Meyer, W., T. G. Mitchell, E. Z. Freedman, and R. Vilgalys. 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *J. Clin. Microbiol.* 31:2274-2280.
- Mochizuki, T., N. Sugie, and M. Uehara. 1997. Random amplification of polymorphic DNA is useful for the differentiation of several anthropophilic dermatophytes. *Mycoses* 40:405-409.
- Mochizuki, T., S. Watanabe, and M. Uehara. 1996. Genetic homogeneity of *Trichophyton mentagrophytes* var. *interdigitale* isolated from geographically distant regions. *J. Med. Vet. Mycol.* 34:139-143.
- Sampaio, J. P., M. Gadanho, S. Santos, F. Duarte, C. Pais, A. Fonseca, and J. W. Fell. 2001. Polyphasic taxonomy of the genus *Rhodospodidum*: *Rhodospodidum kratochvilovae* and related anamorphic species. *Int. J. Syst. Evol. Microbiol.* 51:687-697.
- Simpanya, M. F. 2000. Dermatophytes: their taxonomy, ecology and pathogenicity. In R. K. S. Kushawaha and J. Guarro (ed.), *Biology of dermatophytes and other keratinophilic fungi*. Revista Iberoamericana de Micología, Bilbao, Spain.
- Thanos, M., G. Schöniar, W. Meyer, C. Schweynoch, Y. Gräser, T. G. Mitchell, W. Presber, and H.-J. Tietz. 1996. Rapid identification of *Candida* species by DNA fingerprinting with PCR. *J. Clin. Microbiol.* 34:615-621.
- van Burik, J., R. Schreckhise, T. White, R. Bowden, and D. Myerson. 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Med. Mycol.* 36:299-303.
- Weill, F. X., V. Bernier, J. Maleville, V. Amathieux, F. Claverie, N. Mihalikova, F. Djossou, B. Félix, B. Couprie, and A. Taieb. 1999. Épidémie de teignes du cuir chevelu à *Microsporum audouinii* var. *langeronii* dans un groupe scolaire bordelais. *J. Mycol. Méd.* 9:52-56.
- Weitzman, I., and R. C. Summerbell. 1995. The dermatophytes. *Clin. Microbiol. Rev.* 8:240-259.