Multilocus differentiation of the related dermatophytes Microsporum canis, Microsporum ferrugineum and Microsporum audouinii

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Microsporum ferrugineum, an uncommon causative agent of dermatophytosis, has restricted endemicity. Iranian strains suspected to be M. ferrugineum from two patients with tinea were analysed using the rDNA internal transcribed spacer (ITS) region and the partial β-tubulin (BT2) and translation elongation factor 1-α (TEF1) genes. Strains were compared to reference strains to differentiate M. ferrugineum from its relatives Microsporum canis and Microsporum audouinii. Inter-species differences for TEF1 and BT2 were found to be higher than for the ITS region, which is the current molecular standard for species identification in dermatophytes. Intra-species variation was zero for each of the markers. In silico analysis showed that the restriction enzymes BanI and BshNI were together sufficient to differentiate the three species based on TEF1, whereas a two-step digestion was needed with BT2 or the ITS region. The prevalence of M. ferrugineum in clinical samples in Iran appeared to be higher than suspected on the basis of routine phenotypic identification.

INTRODUCTION

Microsporum canis, Microsporum ferrugineum and Microsporum audouinii are three phylogenetically closely related dermatophytes in the Arthroderma otae complex (Gräser et al., 2008). They have different ecological niches: the former two are anthropophilic, whilst the latter is a zoophilic species. However, all are involved in human infections, and can cause similar clinical manifestations and be transmitted via human-to-human or animal-to-human routes. Identification/differentiation of these related species is important from an epidemiological point of view.

M. ferrugineum has limited endemicity in the Balkans, the Middle East, East Asia and Nigeria (Kane et al., 1997; Weitzman & Summerbell, 1995; Wisuthsarewong et al., 1996). In Iran, this species is rare (Mahmoudabadi, 2006). Routine identification of this species is based on the macroscopic features of colonies on Sabouraud’s glucose agar with cycloheximide and chloramphenicol (SCC) or on potato dextrose agar. Physiological criteria involve urease production, hair perforation and the response to bromocresol purple/milk solids/glucose agar (Ellis et al., 2007; Kane et al., 1997; Weitzman & Summerbell, 1995). Microscopy is limited because microconidia are absent. The most important feature is the production of hyphae with prominent cross-walls (‘bamboo hyphae’; Kane et al., 1997; Ishizaki et al., 2003). Chlamydospores and racket hyphae are commonly observed, and occasionally macroconidia are present. However, phenotypic identification remains difficult because colonies may be similar to atypical (dysgonic) variants of M. canis (Ellis et al., 2007; Gräser et al., 2008). In addition, long incubation times are needed (7–14 days) for characteristics to appear, and these characteristics may also be unstable (Ates et al., 2008; Ninet et al., 2003). Moreover, identification of uncommon dermatophytes outside endemic areas may be difficult due to a lack of experience among microbiologists.
Recently, rapid molecular diagnostics tools have become available for dermatophytes, particularly sequencing of the rDNA internal transcribed spacer (ITS) region (Makimura et al., 1999; Woodgyer, 2004) and PCR-RFLP (De Baere et al., 2010; Jackson et al., 1999). The present paper reports on an 18-month screening study of dermatophytoses in

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**BT2**
Tehran, Iran, where >700 isolates were identified using ITS-RFLP with MvaI. Two dermatophyte strains were encountered that had the RFLP profile characteristics of *M. canis* and *M. ferrugineum* but phenotypically were suspected to be *M. ferrugineum*. This prompted a study of the identification of *M. ferrugineum* and its close relatives, *M. canis* and *M. audouinii*. We analysed sequences of the rDNA ITS region and partial sequences of the *β*-tubulin (*BT2*) and translation elongation factor 1-α (*TEF1*) genes with a view to reliable discrimination of these species.

**METHODS**

**Strains.** Two dermatophytes suspected to be *M. ferrugineum* were isolated from patients with tinea pedis and ectothrix-type tinea capitis. The strains were compared with 30 clinical strains identified morphologically as *M. canis* and originating from tinea capitis (*n* = 5), tinea corporis (*n* = 21), tinea cruris (*n* = 2) and tinea faciei (*n* = 2) infections, and with 10 reference strains (*M. canis*: NBRC 9182, CBS 132.88 and CBS 277.62; *M. ferrugineum*: CBS 457.80, NBRC 6081 and NBRC 5831; *M. audouinii*: NBRC 6074, CBS 280.63, CBS 119448 and CBS 332.68). Strains were cultured on SCC (Oxoid) and incubated at 28 °C for 2 weeks.

**DNA extraction.** Genomic DNA was prepared using methods described previously (Makimura et al., 1999). Briefly, small amounts of young colonies were placed in 1.5 ml tubes containing 200 μl lysis buffer [200 mM Tris/ HCl (pH 7.5), 25 mM EDTA, 0.5% SDS, 250 mM NaCl] and crushed with a conical grinder. Samples were incubated for 20 min at 100 °C and mixed with 150 μl 3.0 M sodium acetate, kept at −20 °C for 10 min and centrifuged at 12 000 g for 10 min. Supernatants were extracted once with phenol: chloroform: isoamyl alcohol (25:24:1) and subsequently with chloroform. The DNA was precipitated with an equal volume of 2-propanol, washed with 300 μl 70% ethanol, dried and suspended in 50 μl ultrapure water. One microlitre of the final suspension was used as template in each PCR.

**Primers and amplification conditions.** The following primer sets were used: the universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) (White et al., 1990) for amplification of the ITS region; the universal fungal primers T1 (5’-AACATGCGTGAGATTGTAAGT-3’) (O’Donnell & Cigelnik, 1997) and Bt2b (5’-ACCCCTCAGTGTAGTGACCCTTGGC-3’) (Glass & Donaldson, 1995) for partial amplification of the *BT2* gene; and the pan-dermatophyte primers EF-DermF (5’-CACATTAACTTGGTCGTTATCG-3’) and EF-DermR (5’-CATCCTTGGAGATACCAGC-3’), designed in this study, for partial amplification of the *TEF1* gene. All primers were synthesized by Sigma-Aldrich. The PCR mixture was prepared using a commercial kit (Takara Bio) and contained 2.5 μl 10× reaction buffer, 200 μM dNTPs, 1 U Tag DNA polymerase, 30 pmol each primer and 1 μl DNA template in a final volume of 25 μl. Reaction mixtures were pre-heated to 94 °C for 6 min and PCR was performed as follows: 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C, with a final extension at 72 °C for 10 min and cooling at 4 °C. The thermal conditions, except for annealing temperatures, were the same for all three markers.

**Sequencing.** PCR products were purified using a QIAquick purification kit (Qiagen) and an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), and sequenced using an automated DNA Sequencer (ABI Prism 3730 Genetic Analyzer; Applied Biosystems).
Sequence analysis. Forward and reverse sequences were edited using MEGA4 (Tamura et al., 2007) and Geneious (www.geneious.com) software. Trimmed ITS sequences were compared with similar sequences in the open access validated CBS database of dermatophytes (www.cbs.knaw.nl/dermatophytes). Inter- and intra-species differences were evaluated using BioEdit (www.mbio.ncsu.edu/bioedit) or Geneious software. Levels of sequence diversity (D) in each target were calculated pairwise using the formula $D = 1 - (M/L)$ (Chilton et al., 1995), in which $M$ is the number of shared positions and $L$ is the total number of alignment positions compared. Restriction sites and fragment sizes were predicted in silico and appropriate restriction enzymes for RFLP analysis were selected using DNAsis software (Hitachi DNAsis MAX version 3.0).

RFLP. ITS region PCR products of all standard and clinical strains, except for $M$. audouinii strains that were identified during an initial ITS RFLP screening with $MvaI$, were digested in 15 μl reaction volumes containing 0.5 μl (5 U) of the enzyme $MaeIII$ (Roche), 1.5 μl buffer (supplied by the manufacturer), 5 μl PCR product and 8 μl molecular-grade water. Reactions were incubated at 55 °C for 120 min.

Electrophoresis. A total of 5 μl ampiclon and 8 μl digested DNA were separated by electrophoresis on a 1.5 and 2% agarose gel, respectively. TAE buffer (40 mM Tris/acetate (pH 8.0), 2 mM EDTA) was used for preparing the gel and for electrophoresis. A 100 bp DNA ladder was used as a molecular size marker. The DNA bands were stained with 0.5 μg ethidium bromide ml$^{-1}$, visualized by UV transillumination and photographed.

RESULTS AND DISCUSSION

PCR amplification of the three target regions yielded single bands of 720–770 bp in all clinical and reference strains. The band sizes for the three species were almost the same for each of the ITS regions and the BT2 and TEF1 genes. Sequence analysis indicated that the DNA products amplified by primers ITS1/ITS4, T1/Bt2b and EF-DermF/EF-DermR were 737, 770 and 720 bp for $M$. canis and $M$. ferrugineum, respectively, and 734, 771 and 721 bp for $M$. audouinii (Fig. 1). Inter-species differences for TEF1 and BT2 were higher than for the ITS region. Whilst the mean nucleotide difference between the three tested species was 5.33 nt for the ITS region, it was 9.33 and 10.67 nt for TEF1 and BT2, respectively (Table 1). No intra-species variation among strains of each species and each marker was seen (data not shown).

In preliminary screening of the tested dermatophytes using ITS region digestion by $MvaI$, all four reference strains of $M$. audouinii yielded a specific RFLP pattern (441, 162 and 131 bp) and were excluded from the following process, whilst all strains of $M$. canis and $M$. ferrugineum produced identical restriction fragments of 441, 165, 103 and 28 bp. An appropriate restriction enzyme was then selected for an RFLP assay for identification of $M$. canis and $M$. ferrugineum by analysing the ITS sequences of 38 strains that were identified as $M$. canis and $M$. ferrugineum, supplemented by sequences from GenBank. Sequences were subjected in silico to 610 restriction enzymes included in the DNAsis software. Only $MaeIII$ proved to be suitable for differentiation of the two species. This enzyme had a single cutting site in $M$. canis, yielding bands of 527 and 210 bp, whereas restriction sites were absent from $M$. ferrugineum and $M$. audouinii and their profile was identical to the original PCR product. Following digestion, the band patterns exactly matched the expectations from in silico analysis. Comparison of ITS sequences with the ITS RFLP with $MaeIII$ confirmed the identities of the clinical isolates from Iran as $M$. canis and $M$. ferrugineum (Fig. 2). Clinical isolates and reference strains of $M$. canis produced white to cream-coloured colonies with a dense cottony surface and yellow to yellow/orange pigments exuding into the SCC medium after incubation for 1 week at 28 °C. In microscopic preparations, spindle-shaped, thick-walled macroconidia with terminal knobs characteristic of $M$. canis were observed. The reference strains of $M$. ferrugineum and the two clinical isolates were waxy to glabrous in texture, slightly raised and folded at the centre, with a cream- to buff-coloured surface and without pigments on the reverse side of the colony (Fig. 3a, b). Microscopic examination revealed bamboo-like and racket hyphae, whilst macro- and microconidia were absent (Fig. 3c, d). The reference strains of $M$. audouinii produced flat, spreading, cream-coloured colonies with a densely cottony surface. The reverse side of the colony was brownish yellow. Macroconidia were absent, but a few pyriform microconidia were observed.

Computer analysis of the TEF1 sequences indicated that restriction enzymes $BanI$ and $BshNI$ could differentiate the three species in a single reaction. These enzymes yielded electrophoretic patterns comprising bands of 610, 78 and 32 bp for $M$. canis, 721 bp (no restriction site) for $M$. ferrugineum and 643 and 78 bp for $M$. audouinii. In silico digestion of the BT2 gene showed that, like the ITS region, a two-step RFLP assay was needed to differentiate the three species (data not shown).

Conventionally, dermatophytes including $M$. ferrugineum are identified by the macro- and microscopic morphology of the colony, supplemented with physiological tests (Kane et al., 1997; Weitzman & Padhye, 1996; Weitzman & Summerbell, 1995). However, tests are time-consuming and require

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>$M$. canis/ $M$. ferrugineum</th>
<th>$M$. canis/ $M$. audouinii</th>
<th>$M$. ferrugineum/ $M$. audouinii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS region</td>
<td>0.3% (2 nt)</td>
<td>1% (7 nt)</td>
<td>1% (7 nt)</td>
</tr>
<tr>
<td>TEF1</td>
<td>1.4% (10 nt)</td>
<td>1.1% (8 nt)</td>
<td>1.4% (10 nt)</td>
</tr>
<tr>
<td>BT2</td>
<td>1.6% (12 nt)</td>
<td>1.9% (14 nt)</td>
<td>0.8% (6 nt)</td>
</tr>
</tbody>
</table>
special skills for reliable diagnosis (Arabatzis et al., 2007; De Baere et al., 2010), particularly in species with a degenerate appearance such as *M. ferrugineum*. Recently, molecular techniques have become available (Kanbe, 2008). Currently, the sequence database of the rDNA ITS region is considered the gold standard for dermatophytes (Gräser et al., 2008; Makimura et al., 1999).

*M. canis*, *M. ferrugineum* and *M. audouinii* are three phylogenetically closely related dermatophytes in the *A. otae* complex (Gräser et al., 2008). In the present study, we focused on two genetic markers other than the ITS region to facilitate differentiation of these species. The ITS regions of *M. ferrugineum* and *M. canis* were highly similar, differing by just 2 bp at one site in ITS2 (Fig. 1), whilst *M. audouinii* differed from these two species in five locations (7 bp) in both ITS1 and ITS2 (Fig. 1). With *Mva*I digestion of the ITS region, the reference strains of *M. audouinii* produced a specific profile, whereas the profiles of *M. ferrugineum* and *M. canis* were identical to each other. These results were in accordance with those of Leon-Mateos et al. (2006), who were unable to find an enzyme differentiating *M. ferrugineum* and *M. canis*; however, we found that digestion of the ITS region with *Mae*III enabled simple PCR-RFLP differentiation of these two species.

We also characterized partial sequences of the **BT2** and **TEF1** genes encoding β-tubulin and translation elongation factor 1-α for *M. canis*, *M. ferrugineum* and *M. audouinii*. Several...
recent studies have shown that β-tubulin is a good target for identification of opportunistic moulds such as *Aspergillus, Penicillium, Scedosporium* and *Phaeoacremonium* (Balajee et al., 2009; Essaki et al., 2008; Gilgado et al., 2005; Mostert et al., 2005; Serra & Peterson, 2007; Staab et al., 2009). It is also well documented that translation elongation factor is the best marker for species identification in *Fusarium* (Geiser et al., 2004; O’Donnell, 2000). Partial sequences of the *BT2* and *TEF1* genes of *M. canis, M. ferrugineum* and *M. audouinii* provided evidence that these loci are more useful and promising than the ITS region. The differences between *M. canis* and *M. ferrugineum* were 12 and 10 nt for *BT2* and *TEF1*, respectively, compared with 2 nt differences in the ITS region (Table 1). Comparing *M. canis* and *M. audouinii*, significant distances of 14 and 8 nt were found in *BT2* and *TEF1*, respectively, with 7 nt in the ITS region. Similarly, the sequence variation between *M. ferrugineum* and *M. audouinii* in *BT2* and *TEF1* was considerable. Thus, *BT2* and *TEF1* are recommended as novel DNA tools for distinguishing closely related species of the *A. otae* complex and perhaps also for other dermatophyte species.

*M. ferrugineum* is a primary cause of prepubertal tinea capitis among oriental races and of inflammatory infections in Caucasians (Kane et al., 1997; Wisuthsarewong et al., 1996). It is a rare causative agent of dermatophytosis in Iran and was reported from Isfahan in the centre (Chadeganipour et al., 1997) and Ahvaz in the south-western part of the country (Mamoudabadi, 2006). All isolates in both reports were from tinea capitis except for one that originated from tinea faciei, and had been identified by conventional phenotypic methods. Our isolates phenotypically matched the ones in those reports and were confirmed by molecular methods. In the anamnesis of the two patients in our study affected by *M. ferrugineum*, there was no evidence of recent travel to areas outside Tehran. The exact prevalence of this species remains to be determined.

*M. canis* is one of the major pathogenic dermatophytes causing tinea capitis worldwide (Aly et al., 2000; Patel & Schwartz, 2011). The clinical strains of *M. canis* used in the present study were obtained from an epidemiological survey in Tehran (unpublished data). Earlier reports stated that *M. canis* is the most common causative agent of tinea capitis in Iran (Mahmoudabadi, 2005; Pakshir & Hashemi, 2006). *M. audouinii* is one of the species causing ectothrix-type tinea capitis, especially among school children, in European and African countries (Patel & Schwartz, 2011; Vella Zahra & Vella Briffa, 2003). This species has not been documented from Iran, nor was it found in the present survey.

In conclusion, species identification of strains in the *A. otae* complex can be reliably performed by partial sequencing of the *BT2* or *TEF1* genes, as well as the ITS region. For rapid screening by RFLP analysis, *TEF1* is the best DNA marker because a single reaction assay is sufficient, whilst a two-step RFLP is required for the rDNA ITS region and *BT2*. *M. ferrugineum* is not a very rare dermatophyte in Iran.

**REFERENCES**


Jackson, C. J., Barton, R. C. & Evans, E. G. (1999). Development of primer sets for the anamnesis of the two patients in our study affected by *M. ferrugineum*, there was no evidence of recent travel to areas outside Tehran. The exact prevalence of this species remains to be determined.

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