ORIGINAL ARTICLE

Identification of *Malassezia* species from patient skin scales by PCR-RFLP

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Objective This study was aimed at the development of a DNA-based procedure directly applicable to pathological skin scales and at the assessment of its value in rapid laboratory confirmation and identification of each of the seven *Malassezia* species. These lipophilic basidiomycetous yeasts in predisposed individuals are involved in pityriasis versicolor, seborrheic dermatitis, blepharitis, folliculitis, atopic dermatitis and fungemia. Standard identification procedures to species level are available, but so far no system for direct detection and characterization of *Malassezia* species in clinical specimens is available.

Methods *Malassezia* DNA was extracted from pathological skin scales by a modified hexadecyltrimethylammonium bromide (CTAB) method and amplified by single and nested polymerase chain reaction (PCR), assays using the general fungal ITS 1/4 and 3/4 primers for amplification of sequences from the *Malassezia* major ribosomal DNA complex. Restriction fragment length polymorphism (RFLP) analysis of PCR products was used in subsequent species identification. DNA extracted from culture-positive skin scales was also tested by PCR and the RFLP patterns obtained were analyzed.

Results A total of 36 isolates were tested. Distinct pure culture and skin-scale ITS 3/4 *Hinf*I and *Alu*I restriction patterns differentially identified *M. furfur*, *M. globosa*, *M. restricta*, *M. sympodialis*, *M. pachydermatis*, *M. obtusa* and *M. slooffiae*. *Malassezia* DNA was extracted from pathological skin scales and RFLP identified solitary and multiple *Malassezia* species in the same specimen. Molecular identification was confirmed by cultures and biochemical tests. Concurrent detection and identification of *Candida* and *Yarrowia* species was also feasible from skin scales.

Conclusion The proposed method, described for the first time, could provide a sensitive and rapid detection and identification system for *Malassezia* species, which may be applied to epidemiological surveys and routine practice.

Keywords *Malassezia* infections, PCR identification, skin scales

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INTRODUCTION

For many years the nomenclature and taxonomic status of the genus *Malassezia* has been controversial. This was recently resolved and four new species (*M. globosa*, *M. obtusa*, *M. slooffiae* and *M. restricta*) were incorporated into the genus, which originally included only three species (*M. furfur*, *M. pachydermatis* and *M. sympodialis*) [1]. Yeasts of the genus *Malassezia* inhabit healthy and diseased skin in sites rich in sebaceous glands such as the thorax, the interscapular region and the forehead. Apart from causing pityriasis versicolor

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Malassezia species have been implicated in seborrheic dermatitis and blepharitis, folliculitis, dandruff and atopic dermatitis [2] although the pathogenetic relationship is obscure. It has also been reported that the non-lipid-dependent *M. pachydermatis* is implicated in epidemics of folliculitis in intensive care unit patients, possibly acquired from fomites [3,4].

As two of the new *Malassezia* species, *M. obtusa* and *M. restricta*, are difficult to grow, the isolation rate of individual species in culture may not be representative of the actual flora, casting doubt on possible species-specific pathology. Due to the lipid dependence of the organism, sugar fermentation tests are not appropriate for species differentiation. At present, all species are identified according to colony morphology, microscopic characteristics and by time-consuming and complicated lipid assimilation tests [5,6]. Identification by these conventional tests can be confirmed by elaborate and costly methods of no direct diagnostic significance, such as pulsed field gel electrophoresis (PFGE), recombinant RNA sequencing and nuclear DNA comparisons [7,8]. Conversely, the less costly and more rapid PCR-based method of random amplification of polymorphic DNA (RAPD) has been frequently used to investigate epidemics, as in a neonatal intensive care unit [3]. In light of the intricacy of interpreting the biochemical tests required for characterizing each species of human and veterinary origin [9], a direct and economical method for the detection and identification of the *Malassezia* species would be a useful diagnostic tool in veterinary mycology and in epidemiological surveys. However, detection and identification of *Malassezia* species DNA by direct tests on skin scales from pathological specimens has not yet been reported.

The aim of this study was two-fold. Firstly, we planned to build up a 'library' of restriction endonuclease patterns of *Malassezia* species amplification products generated by the internal transcribed spacer (ITS) 3/4 primers – which amplify the 5.8S and part of the 28S region of the major nuclear ribosomal DNA complex. Secondly, we intended to use the restriction 'library' data in the development of a rapid PCR-based method for testing small quantities of patient skin scales so as to detect directly and confirm the identity of *Malassezia* species.

MATERIALS AND METHODS

Strains and patients

A total of 36 strains consisted of five Type strains (*M. sympodialis* CBS 7222; *M. slooffiae* CBS 7956; *M. globosa* CBS 7966; *M. obtusa* CBS 7876; *M. restricta* CBS 7877), two Neotype strains (*M. furfur* CBS 7019; *M. pachydermatis* CBS 1879), two Reference strains (*M. furfur* CBS 6001; *M. furfur* Institute Pasteur – IP 1363-82) and 20 clinical strains isolated from lesions of scalp, neck and trunk [*M. furfur* (three strains), *M. sympodialis* (four strains), *M. slooffiae* (two strains), *M. restricta* (three strains), *M. pachydermatis,* isolated from ear lesions of a dog (one strain) and *M. globosa* (seven strains)] (Table 1). In addition, more clinical isolates were

Table 1 Dermatological condition and isolation of *Malassezia* clinical strains from different anatomical sites

	***Malassezia* species**	**Skin disease**	**Sampled region**
1.	*M. furfur* and *M. globosa*	Pityriasis versicolor	Interscapular region
2.	*M. furfur*	Pityriasis versicolor	Sternum
3.	*M. furfur*	Pityriasis versicolor	Back
4.	*M. sympodialis*	Pityriasis versicolor	Interscapular region
5.	*M. sympodialis* and *M. globosa*	Pityriasis versicolor	Back
6.	*M. sympodialis*	Pityriasis versicolor	Chest
7.	*M. sympodialis*	Pityriasis versicolor	Neck
8.	*M. restricta* and *M. globosa*	Pityriasis versicolor	Back
9.	*M. restricta*	Seborrheic dermatitis	Head
10.	*M. restricta*	Seborrheic dermatitis	Head
11.	*M. slooffiae*	Seborrheic dermatitis	Head
12.	*M. slooffiae*	Seborrheic dermatitis	Mid-eyebrow region
13.	*M. globosa*	Seborrheic dermatitis	Pre-auricular region
14.	*M. globosa*	Pityriasis versicolor	Neck
15.	*M. globosa*	Pityriasis versicolor	Sternum
16.	*M. globosa*	Pityriasis versicolor	Interscapular region
17.	*M. pachydermatis*	Dog otitis	External ear

included, comprising strains of *M. sympodialis* (two strains), *M. globosa* (two strains), *M. obtusa* (two strains) and *M. pachydermatis* (one strain), donated by Gillian Midgley (St. John's Institute of Dermatology, St. Thomas Hospital, London, UK).

The Hospital Ethics Committee approved the protocol, and the skin-scale samples were taken following informed patient consent.

Identification procedures

Conventional identification to species level was achieved as previously described [5,6]. Briefly, *M. restricta* was identified by the negative catalase test. In order to exclude the remote possibility of the presence of a rare *M. pachydermatis* in human skin, isolates were also tested for their ability to grow, in subsequent subcultures, on a lipid-free medium. The other species were identified according to their microscopic characteristics and their ability to assimilate Tween-20, -40 and -80 and Cremophor El [6] (Sigma, MO, USA). Esculin hydrolysis tests were also employed to confirm the identity of *M. sympodialis*.

Other yeasts, such as *Candida* and *Yarrowia* species, which were simultaneously isolated with *Malassezia* species in cultures of skin scales, were identified according to their carbohydrate assimilation profile by the API ID 32C system (BioMérieux, Marcy l' Etoil, France), followed by confirmatory additional physiological and morphological tests.

Antifungal susceptibility testing

Only *Yarrowia* (*Candida*) *lipolytica* susceptibility tests were performed, because to our knowledge this is its first report as a *Malassezia* co-isolate from human lesions. The minimum inhibitory concentrations (MIC) of amphotericin B, 5-flucytosine, itraconazole, fluconazole, ketoconazole and voriconazole were determined against three *Y. lipolytica* strains , one each from seborrheic dermatitis of the scalp, face and trunk, and two from candidemia cases, by the NCCLS microdilution reference method for yeasts [10] with *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 used as Quality Control strains and *C. albicans* ATCC 90028 as a Reference strain. The MIC_{100} was optically read for amphotericin B, and MIC_{80} was read for all other antifungals after 48 h incubation at 28–30 °C in five independent single colony-forming unit (CFU) assays, and the range of MIC values for *Y. lipolytica* was recorded.

DNA extraction from pure cultures

All reference strains and clinical isolates were grown and maintained on modified Dixon's agar (3.6% malt extract agar, 0.6% mycological peptone, 1% agar No. 1, 2% ox bile, 1% Tween-40, 0.2% glycerol (all from Oxoid, Basingstoke, UK) and 0.2% oleic acid), at 32 °C for 8 days. The requirement for lipid-containing media resulted in either frequent failure to extract DNA from pure culture, or a very poor DNA yield. Therefore, to achieve adequate yeast cell lysis and sufficient DNA purity for PCR amplification, a modification of the method proposed by the manufacturers of the Silica extraction kit (MBI, Fermentas, Lithuania) was incorporated into the extraction protocol as follows. A standard loopful of volume capacity 10^{-3} (Greiner GmbH, Germany) of yeast pure culture was suspended in 100 µl lysis buffer [(200 mM Tris–HCl, pH 8, 250 mM NaCl, 25 mM ethylenediamine-tetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS) (all from Sigma)] as previously described [11]. Cells were disrupted mechanically with an orbital homogenizer, following addition of a further 350 µl lysis buffer. DNA was extracted once by suspending the homogenate in an equal volume of phenol : chloroform : isoamyl alcohol (vol : vol : vol, 25 : 24 : 1). After 60 min centrifugation at 4 °C and 13 680 *g*, the upper aqueous layer was transferred into a new microcentrifuge tube, mixed with an equal volume of molecular grade phenol (Sigma) and centrifuged at room temperature at 22 570 *g* for 10 min. The extraction method suggested by the manufacturer was then applied to the supernatant.

Sensitivity of the CTAB extraction method

Sensitivity was determined using pure cultures as described previously [11,12].

DNA extraction from scalp, neck and trunk skin scales

Skin scales from 11 patients (three with scalp and interscapular seborrheic dermatitis and

eight with trunk pityriasis versicolor) were collected and transferred to the laboratory sealed between two microscope slides. A portion of the pathological material was microscopically examined, and following confirmation of the presence of the yeast cells, a quantity of the remaining skin scales was inoculated on Dixon's agar, while a loopful of the remaining scales where collected with a disposable standard 10^{-3} inoculation loop (greiner), moistened in sterile phosphate-buffered saline to facilitate adherence of the skin scale, and processed for DNA extraction.

No skin scales from healthy individuals were used, as at this stage the aim of the study was to evaluate the potential of the extraction/identification method from pathological skin scales and not from sebum, as would have been the case if healthy individuals with no scaly lesions had been tested.

Malassezia DNA was extracted using a modification of the CTAB method as previously described [12]. This strong cationic detergent was selected as a DNA carrier because it has been traditionally very effectively used in lysing robust chitinized tissues and it is highly sensitive in nucleic acid isolation from small samples [12], as in the case of attempting to extract DNA from a small amount of skin scales. Briefly, the skin scales were suspended in 100 µl buffer [5% CTAB (Sigma); 700 mM Tris–HCl, pH 8; 10 mM EDTA; and 5% 2-mercaptoethanol]. Skin scales and yeast cells were mechanically disrupted for 3 min in an orbital homogenizer. To the homogenate, 400 µl of CTAB buffer was added and following incubation for 30 min at 65 °C with occasional swirling, chloroform : isoamyl alcohol (vol : vol, 24 : 1) extraction was performed. Following centrifugation at 8000 *g* for 10 min at room temperature the aqueous phase was obtained and nucleic acids were precipitated with an equal volume of cold isopropanol by centrifugation at room temperature and 8000 *g* for 10 min. The pellet was washed in 500 µl wash buffer (76% ethanol, 10 mM ammonium acetate) by standing at room temperature, for 15 min. DNA was precipitated after centrifugation at room temperature and 8000 *g* for 10 min. The wash buffer was removed carefully, so as not to loosen the pellet, which was allowed to dry briefly for 2–4 min before washing the DNA in 70% ethanol to collect the nucleic acids by centrifugation as before. The pellet was dried and resuspended in 30–40 µl sterile double-distilled water.

Primers and hot-start PCR reaction conditions

The general fungal ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′), ITS 3 (5′-GCATCGATGAAGAACGCAGC-3′), and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (Interactiva, GmbH, Germany), as defined by White et al. [13], were used to amplify *Malassezia* species sequences. Each amplified sequence contains flanking parts of two of the three rRNA genes (18S, 5.8S and 28S and either or both of the ITS 1 and 2 regions (which flank the 5.8S rRNA gene) and hold enough variation to define given species. The target DNA was extracted from pure cultures and from patient skin scales obtained from lesions. The 50 µl of the PCR master mix contained 6 µl $MgCl_2$ (25 mM), 10 µl Mg^{2+}-free buffer (100 mM Tris–HCl, 500 mM KCl and 1% Triton-X), 2.5 U Taq Polymerase (Promega, Madison, WI, USA), 0.04 mM of each dNTP (CLONTECH, Palo Alto, CA, USA), and 5 µl of template DNA.

The PCR reactions were performed in a PCR thermocycler (Robocycler, Gradient 40, Stratagene, USA) at 30 repetitions of the cycle: 1 min at 95 °C, 1 min at 55 °C, and 1 min 30 s at 72 °C. The final cycle comprised 1 min at 95 °C, 1 min at 55 °C and 5 min at 72 °C.

Nested PCR reactions

In cases where the ITS 3/4 amplification product was weak and therefore not sufficient for restriction endonuclease analysis, the template *Malassezia* DNA from diseased skin scales was used for amplification with the ITS 1/4 primers [13] under the aforementioned PCR conditions. The product was then subjected to nested PCR using the ITS 3/4 primers.

Sequencing of PCR products

The amplification products of the five Type strains (*M. sympodialis* CBS 7222; *M. slooffiae* CBS 7956; *M. globosa* CBS 7966; *M. obtusa* CBS 7876; *M. restricta* CBS 7877) and the two Neotype strains (*M. furfur* CBS 7019; *M. pachydermatis* CBS 1879) were sequenced by Comfort Read®, MWG-BIOTECH AG (Edersberg, Germany) and a panel of restriction endonucleases was tested,

so as to investigate whether the different species would be differentiated from distinct RFLP patterns.

Restriction fragment length polymorphism (RFLP) analysis

The concentration of each ITS 3/4 amplification product was estimated following brightness comparison with the *Msp*I digest of the *pBR*322 DNA molecular marker and 1 μg was digested with 30 U each of *Alu*I, *Hinf*I and *Msp*I restriction endonucleases (New England Biolabs, Beverly, MA, USA) at a total volume of 30 μl in the supplied buffer, and incubated at 37 °C for 2–16 h. Restriction fragments were visualized under a UV transilluminator (Herolab, E.A.S.Y., Weisloch, Germany) in 2.5–3% ethidium bromide-stained standard agarose gels.

Reproducibility and stability of *Malassezia* species populations

To test the stability and reproducibility of the extraction method, and of the subsequent PCR-RFLP results, a mean of five separate DNA extractions were performed from serial subcultures of *Malassezia* Type, Neotype, Reference and clinical isolates. In addition, DNA extractions and subsequent amplification reactions were carried out from non-viable *Malassezia* cells from old cultures.

Multiple DNA extractions were also performed from pure cultures of *C. albicans* strains (50 strains), *C. dubliniensis* (20 strains), *C. glabrata* (50 strains), *C. parapsilosis* (50 strains) and *C. tropicalis* (50 strains) to test whether yeast species, residing on human skin, can be distinguished from *Malassezia* species, either by their amplicon size or by their characteristic restriction patterns. In addition, a total of five clinical *Y.* (*Candida*) *lipolytica* isolates were tested in multiple trials, as it was noted that *Y. lipolytica*, apart from being a causative agent for candidemia, can be co-isolated in cultures of skin scales from individuals with seborrheic dermatitis. Therefore, it was considered necessary to determine whether its ITS 3/4 amplification products could be differentiated from those of *Malassezia*. DNA from pure cultures of anamorphic basidiomycetous yeasts, such as *Filobasidiella* (*Cryptococcus*) *neoformans* (50 strains), *Trichosporon asahii* (eight strains) and *T. mucoides* (four strains), was also tested with the ITS 3/4 primers.

Table 2 *Malassezia* Type, Neotype and clinical strains RFLP of ITS 3/4 PCR products generated by *Alu*I and *Hinf*I

***Malassezia* species**	**ITS 3/4 amplification product (bp)**	***Alu*I digest (bp)**	***Hinf*I digest (bp)**
M. furfur	509	268, 241	483, 18, 8[a]
M. globosa	430	241, 173, 16[a]	412, 10, 8[a]
M. obtusa	506	NRS	480, 18, 8[a]
M. slooffiae	486	368, 118	403, 83[b]
M. restricta	410	NRS	392, 18, 8[a]
M. sympodialis	374	NRS	356, 10, 8[a]
M. pachydermatis	483	374, 109	457, 18, 8[a]

NRS, no restriction site.
[a]Restriction fragment not visible in standard agarose gels.
[b]Restriction fragment occasionally visible in standard agarose gels.

Relative sensitivity of PCR

The relative sensitivity of the method was determined, as previously described for *Candida* species [12].

RESULTS

Malassezia species identification by PCR

The 5.8S rDNA and the ITS 2 region, amplified from type, neotype, reference and clinical isolates of *Malassezia* species by using the ITS 3 and 4 primers, differed in length among *M. furfur*, *M. sympodialis*, *M. globosa* and *M. pachydermatis* (Table 2). However, *M. restricta* could not always be discriminated from *M. slooffiae* or *M. pachydermatis* in standard agarose gels, because, according to sequencing data of the CBS Type and Neotype strains the amplicons differ by only 76 and 73 bp, respectively (Figures 1 and 2a); however, differentiation among these species was attainable through the *Hinf*I and *Alu*I restriction patterns, visualized in 2.5% standard agarose gels (Figures 2a,b and 3). Likewise, no difference in the 3/4 PCR products was identified for *M. furfur* and *M. obtusa*, in standard agarose gels, as they differed by only 3 bp (Figures 1 and 2a), but species identification was feasible through the ITS 3/4 *Alu*I restriction pattern (Figure 3). No *Hinf*I restriction site was recognized on the *M. furfur* Reference strain CBS 6001 (Figure 1). The noticeable migration of the amplicon following incubation with *Hinf*I

***M.furfur* CBS 7019 ITS 3/4 amplification product of 509 bp**
GAATTGCAGAATTCCGT**GA**ATCATC**GA**ATCTTTGAACGCACCTTGCGCTCCATGGTATTC
CGTGGAGCATGCCTGTTTGAGTGCCGTGAATTCTCTCTCCCCAAGCGGTTGCGATTGCAC
TGCTTTGGCGGACGAGGTTGGATGGGTGCTTCTGCCTGTTTCGCAAGAAACAGGCTCGCC
CGAAATGCATTAGCGCCTTTGGGACACACTCTGCAAACCGCTCTGAAAGGGAGGGCGGCA
GAGGGGATGGAGGAACTCCGCCCGTCAG→CTATACCAAACTTTGCCCCAGGCAAGCGCATG
GCATGATACGTCATTTGCTGTGTGTGCGTCCTGAGGCAAGGGGCCGATGTGGTGCCTTTG
TCACTCTGTGGGTGTGTTGGTGCGCTACCAATCCTGCGTGGAGGCTGGTCTGTGCGCAAG
CATGGAGCAGTTCTTGTGAACGCATTCCCTTTTTCATTTCTGGTCTCAAATCAGGTAGGA
TCACCCGCTGAACTTAAGCATATCAATAA

***M.furfur* CBS 6001 ITS 3/4 amplification product of 479 bp**
CCTTGCGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGCCGTGAATTCTCTCTCC
CCAAGCGGTTGCGATTGCACTGCTTTGGCGGACGAGGTTGGATGGGTGCTTCTGCCTGTT
TCGCAAGAAACAGGCTCGCCCGAAATGCATTAGCGCCTTTGGGACACACTCTGCAAACCG
CTCTGAAAGGGAGGGCGGCAGAGGGGATGGAGGAACTCCGCCCGTCAG→CTATACCAAACT
TTGCCCCAGGCAAGCGCATGGCATGATACGTCATTTGCTGTGTGTGCGTCCTGAGGCAAG
GGGCCGATGTGGTGCCTTTGTCACTCTGTGGGTGTGTTGGTGCGCTACCAATCCTGCGTG
GAGGCTGGTCTGTGCGCAAGCATGGAGCAGTTCTTGTGAACGCATTCCCTTTTTCATTTC
TGGTCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATCAATAAGCGGGAGGAA

***M. globosa* CBS 7966 ITS 3/4 amplification product of 430 bp**
GAATTCCGN**GA**ATCATC**GA**ATCTTTGAACGCACCTTGCGCTCTATGGTATTCCGTAGAGC
ATGCCTGTTTGAGTGCCGTGAATTCTCCCATCCCAAGCGGTTTTTATCAAAGAATTGCTA
GGCGAAGGGGTTGAGATGGGCGTTGTTATAACTGCTTTTCTTCTCTAGAAAAG→CTCGCCC
GAAAAACAG CTAACGCCTCTGGGCCACTTTGCATCCGCTTCTCTGAGGGGAGAAGCGGCC
AAGCGCGCTCTGATCATCAGGCATAGCATGATACGTCATTTGCTATGCTGTAGGAGAGCA
TTTGGTTGTGGTTATACCGCGTGCGTCATTTTTTTTTTTGCAAACGCAAAGAAAAAAGCC
CCCTTTCATTTCTGGTCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATCAATA
AGCGGGAGGA

***M. obtusa* CBS 7876 ITS 3/4 amplification product of 506 bp**
GAATTGCAGAATTCCGT**GA**ATCATC**GA**ATCTTTGAACGCACCTTGCGCTCTATGGTATTC
CGTGGAGCATGCCTGTTTGAGTGCCGTGAATTCTCTCTCCCCAAGCGGTTGCGATTATTG
CGCTGCTTTTGGCGGACGAGGTTGGATGGGTGCTTCTGCCTGTTTCTTGCTAAACAGGCT
CGCCCGAAATGCATTAGCGCCTTTTTGGGACACAATCTCTGCAATACCGCTCCTAAAGGG
AGGGCGGCAGAGAGCGGGGGGGTTCCCACCCGTTAGCCATACCAAACTTTGCCCAGGCAA
GCGCATGGCATGATACGTCATTTGCTGTGTGCGCGCTCTAGGCAAGGGGCCGACGCGGTG
CCTTCTGCACTCTGTGAGTTGTGTTGGTGCGCTACCAATCCTGCGTGGAGGCTGGTCCGT
GCGAGCAGCCTTGTGAACGCATTCCCTTTTTCATTTCTGGTCTCAAATCAGGTAGGATCA
CCCGCCGAACTTAAGCATATCAATAA

***M. restricta* CBS 7877 ITS 3/4 amplification product of 410 bp**
GAATTCCGN**GA**ATCATC**G→A**ATCTTTGAACGCACCTTGCGCTCTATGGCAATCCGTAGAGC
ATGCCTGTTTGAGTGCCATGAAATCTCCCACCCCAAGCGGTTTTTACATGAAACGGCTTG
GCGGATGGGGTCTGGATGGGTGCCTCTGCCTGCGCTACCTAGCACAGGCTCGCCCGAAAT
GCATGAGCGCCTTGAGACACTTTGCATCCGCCTCTCTGTTTGGGAGGAGGCGGCCAAGCA
GTGTTTTTCTCCTGGCATGGCATGATACGTCATTTGCTATGTCGCCTAAAGGAGGAATGT
TTGGTTGTGTCTGCGTGTGCTTCGAACTTGCCTCTGTGGCACATCCCAATTTCACTTCTG
GTCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATCAATAAGCG

***M. sympodialis* CBS 7222 ITS 3/4 amplification product of 374 bp**

GAATTCCGC**GA**ATCATC**GA**ATCTTTGAACGCACCTTGCGCTCCATGGTATTCCGTGGAGC
ATGCCTGTTTGAGTGCCGCGAATTCTCCCTCCCCTTACGGTGGCCGAAAGGCCGAAGTAG
GGCGGACGGGGTAGGATGGGTGTTGCTGCCTGGGGATTGTACCAGGCTCGCCCGAAATGC
ATAAGCGCCAGGACCCTCGCTACCGCTCTCTAGGGAAGAGTGGCTAAGCGACCGCTGAGC
ATGGCATGATACGTCATTTGCTGTGTGTGGGCGGCCGGTTGGAGAGGTGTCTGCTTTACC
AGCCCTTTTTTAATTCTGGTCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATC
AATAAGCGGGAGGA

***M. slooffiae* CBS 7956 ITS 3/4 amplification product of 486 bp**
ATATCAATAAGCGGAGGATGCATCGATGAATAACGCNGNATATCAANATCTCNGAANNCN
CCTTGCGCTCTGTGGTATTCCGCAGAGCATGCCTGTTTGAGTGCCGTGAAATCTCTCCCC
CCAAGTGGTGTTTGTATAGGACGCTACGCGGCGGCGGAGGTTGGATGAGCGTCGTGCCTA
CCGCGCCTTGGTAGGCTCGCCTCGAAAAGCATGAGCGCTTGAGAACACTTGTCGCCTGTG
AGAGGCAGGCGGGCAGAACAGGTGAGCGAATGCTTCTCCTCCGGAGAAGCGCCCACCGGT
CTGCGTGTGCGCATGGCATGATACGTTCCTTGCTATGTGTGGCGCGCAAACCCCATCTGG
GTTAGTAG→CTTCTTGCTTCTGGCGTGATCTCGTGTGCGCTTC**GA**ATCGCACAGCCCTCTC
TTCACTTCTGGCCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATCAATAAGCG
GGAGGA

***M. pachydermatis* CBS 1879 ITS 3/4 amplification product of 483 bp**
GAATTGCAGAATTCCGT**GA**ATCATC**GA**ATCTTTGAACGCACCTTGCGCTCCATGGTATTC
CGTGGAGCATGCCTGTTTGAGTGCCGCGAATTCTCCCACCCCAAACGGTTGCCGAAAGGT
ACTGTGCGGCGGAGGGGTTGGATGGGTGCTACTGCCTGTGGTGGGGAAACTACAACAGGC
TCGCCCGAAATGCATTAGCGCCTCAGGACACATTCGCTACTGCTCTACAAAAGGAAGAGC
AGCGAAGCGTATGGGGGGGAAAACCACCACCGGTTATTCCAAACCTCGCCTGACTGTTTT
TGCGCGCATGGCATGATACGTCATTTGCTGTGTTGCGTAGGTTGGTGCGTGGGACTGTGT
GAGTGCCTCTAGAG→CTTTGAGAAGTGTGTTTCGTGTGCTACGAACTGAAAAAGTACATTC
CTTTTTTTCAATTCTGGTCTCAAATCAGGTAGGATCACCCGCTGAACTTAAGCATATCAA
TAA

Figure 1 Sequences of ITS 3/4 amplification products of *Malassezia* Type, Neotype and Reference CBS strains. *Hinf*I restriction sites are marked in bold type and the *Alu*I sites are marked by arrows.

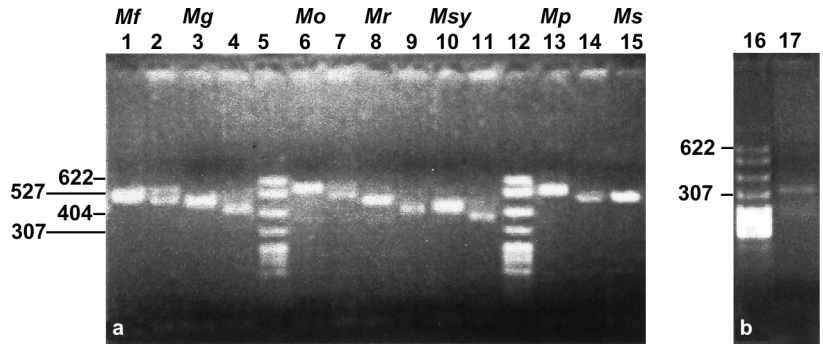

Figure 2 ITS 3/4 amplification products and corresponding restriction fragments of *Malassezia* species from pure cultures in 2.5–3% standard agarose gels. (a) Lane 1, *M. furfur* (*Mf*, CBS 6001); lane 3, *M. globosa* (*Mg*, GM 56); lane 6, *M. obtusa* (*Mo*, CBS 7876); lane 8, *M. restricta* (*Mr*, CBS 7877); lane 10, *M. sympodialis* (*Msy*, CBS 7222); lane 13, *M. pachydermatis* (*Mp*, CBS 1879); lane 15, *M. slooffiae* (*Ms*, CBS 7956). Each *Malassezia* species tested is shown above the corresponding lanes. ITS 3/4 *Hinf*I digests of each *Malassezia* species tested (panels a,b), lane 2, *Mf* (no restriction); lane 4, *Mg*; lane 7, *Mo*; lane 9, *Mr*; lane 11, *Msy*; lane 14, *Mp*; lane 17, *Ms*. Lanes 5, 12 and 16, molecular size marker *Msp*I digest of pBR322 DNA.

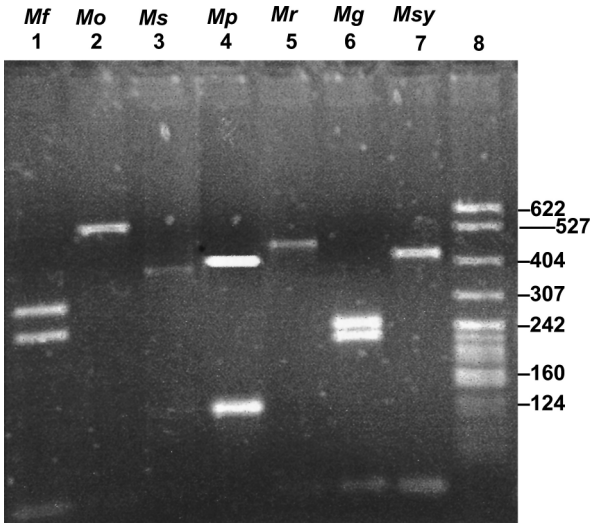

Figure 3 ITS 3/4 *Alu*I digests of *Malassezia* spp. Lane 1, *M. furfur* (*Mf*) clinical strain; lane 2, *M. obtusa* (*Mo*) CBS 7876; lane 3, *M. slooffiae* (*Ms*) CBS 7956; lane 4, *M. pachydermatis* (*Mp*) (dog otitis); lane 5, *M. restricta* (*Mr*) (clinical strain); lane 6, *M. globosa* (*Mg*) (clinical strain); lane 7, *M. sympodialis* (*Msy*) CBS 7222. Lane 8, molecular size marker *Msp*I digest of pBR322 DNA. *M. obtusa*, *M. restricta* and *M. sympodialis* do not possess a restriction site for *Alu*I, while one site is recognized on *M. furfur*, *M. globosa*, *M. pachydermatis and M. slooffiae* differentiating all four of them.

(Figure 2, lane 1) is an occasionally misleading artifact, often visible in standard agarose gels.

Amplified products from five Type and Neotype *Malassezia* strains, namely *M. furfur* CBS 7019, *M. obtusa* CBS 7876, *M. globosa* CBS 7966, *M. pachydermatis* CBS 1879 and *M. restricta* CBS 7877 had two *Hinf*I recognition sites (Figure 1) producing different restriction patterns, which confirmed the identity of each species. Smaller restriction fragments, of 100 bp or less, were not perceptible in 2.5–3% standard agarose gels. However, digestion of amplicons could be visualized, as judged by the relative migration of the resulting larger restriction fragment in the gel (Figure 2a). *M. sympodialis* CBS 7222 Type strain had only one *Hinf*I recognition site (Figure 1), but it produced a clearly smaller product upon amplification using either the ITS 3/4 (Figure 2a) or ITS 1/4 (data not shown) primer pairs. Thus, it was readily differentiated from all the other species by a single PCR.

M. obtusa, with no *Alu*I resticion site, was discriminated from *M. furfur* by its *Alu*I ITS 3/4 distinct restriction pattern (Figure 3). None of the clinical isolates tested produced atypical or ambivalent *Alu*I restriction patterns in comparison with those presented in Figure 3 and all clinical *M. furfur Hinf*I restriction patterns conformed to that of *M. furfur* CBS 7019 (Figure not shown). Also, none of the *Candida* species tested produced *Hinf*I ITS 3/4 restriction patterns confusing the differentiation of *Malassezia* species, as they were dissimilar from those of *Malassezia* and distinct for each *Candida* (figure not shown).

The ITS 3/4 amplification products of the other basidiomycetous genera tested did not match those of *Malassezia* species, as *Filobasidiella* (*Cryptococcus*) *neoformans*, *T. asahii* and *T. mucoides* produced smaller amplicons of approximately 404 and 380 bp, respectively. They were also readily differentiated from those of *Malassezia* by characteristic *Alu*I and *Hinf*I restriction fragments (data not shown).

Reproducibility and stability of *Malassezia* species populations

All the obtained *Malassezia* species-distinguishing patterns from Type, Neotype, Reference and clinical strains were reproducible upon testing DNA from separate extractions and consequent restriction digestions of the amplification products. As expected, molecular identification of non-viable *Malassezia* strains was in accordance with the identification previously obtained by biochemical and molecular methodology. Conventional species classification and identification by the *Hinf*I and *Alu*I ITS 3/4 digestions were found to be in accordance in every independent assay.

Instability of certain *Malassezia* species in cultures was observed on three occasions. Upon 10 consecutive subcultures of primary *M. globosa* and *M. restricta* isolates, the former was outgrown, in two cases by *M. furfur* and *M. sympodialis*, and the latter by *M. globosa*. Despite this, the PCR-RFLP method directly identified the concurrent presence of mixed *Malassezia* species in a single specimen.

Sensitivity of the method

The relative sensitivity, as assayed using pure cultures, was 10 ± 5 CFU.

Susceptibility testing

The MIC ranges against *Y. lipolytica* were amphotercin B, 0.25–0.5 mg/L; flucytosine, 1–16 mg/L; fluconazole, from 8 to >64 mg/L; itraconazole, 0.5–3 mg/L; ketoconazole, 0.25–3 mg/L; and voriconazole, 0.125–0.25 mg/L.

Malassezia species identification from pathological skin scales

DNA extraction, amplification and endonuclease digestion were successful in five of eleven pathological specimens tested. The failure of DNA extractions from the remaining six specimens was attributed to the low yeast load of the skin scales, as determined by microscopy, and subsequently, by the density of growth on Dixon's medium. Three of the five originated from pityriasis versicolor and two from seborrheic dermatitis cases. In the positive extractions from pathological skin scales, the intensity of the ITS 3/4 amplification product was proportional to the yeast load of the skin scales. PCR-RFLP detected and identified *M. restricta* and *M. sympodialis* from two patients with seborrheic dermatitis and *M. globosa* from three patients with pytiriasis versicolor. This was later confirmed by positive culture and subsequent biochemical tests.

In cases where inadequate amounts of PCR 3/4 products were obtained from skin-scale-extracted DNA, nested PCR with ITS 3/4 following PCR with the 1/4 primers was required in order to augment the ITS 3/4 amplification products and render them susceptible to restriction digestion. In all cases, smearing was observed (Figure 4a,b) upon electrophoresis of amplified target DNA from skin scales. DNA purification procedures, which would have improved the appearance of the electrophoresed amplicon, were not undertaken, as this would have further diminished the amount of extracted DNA.

On occasion, two or more PCR amplification products were obtained using DNA extracted from pathological skin scales. This was expected as human skin usually accommodates more than one *Malassezia* species. Again, whenever products of the single PCR assay were weak, nested PCR was required to obtain products sufficient for endonuclease restriction.

As accounted for by the design of the study, a second amplicon (approximately 330 bp) was detected in one specimen of pathological material, suggestive of the presence of a *Candida* species (Figure 4a, lane 3). This amplicon size (approximately 330 bp) was indicative of *C. albicans*, whereas that of 410 bp was indicative of *M. restricta* (Figures 1 and 2a). Molecular identification was consistent with the conventionally isolated and identified *M. restricta* and *C. albicans* from the skin scales of that patient. Subsequent digestion with *Hinf*I confirmed the presence of *Malassezia* and *C. albicans* in the same specimen, as a mixed *Hinf*I restriction pattern was obtained (280 bp), corresponding to *C. albicans* (data not shown) and one representative of *M. restricta* (Figures 1 and 2a). A third amplification product of approximately 242 bp was also concurrently obtained with that of *M. restricta* (Figure 4a). Again, cultures of the pathological material and subsequent biochemical tests confirmed the presence of *Y. lipolytica* (Figure 4c). *Y. lipolytica*, producing an amplicon of approximately 242 bp, no *Alu*I restriction site and two *Hinf*I fragments (approximately 200 and 42 bp each), could be easily discriminated

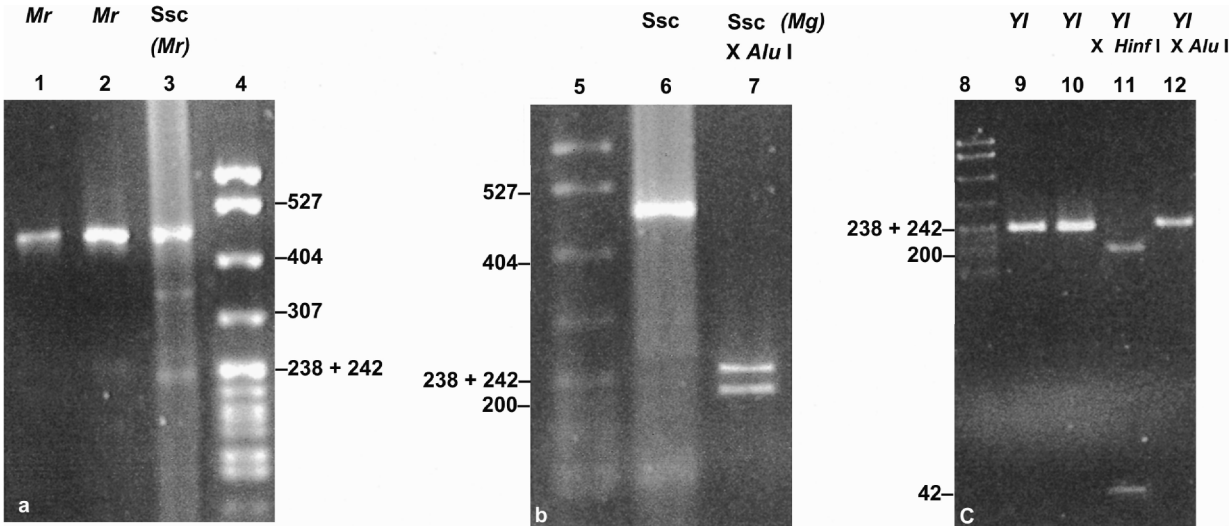

Figure 4 ITS 3/4 direct *Malassezia* and other yeast species detection from seborrheic dermatitis (SD) and pityriasis versicolor (PV) skin scales (Ssc) run in 2.5% standard agarose gels. (a) Lane 1, *M. restricta* from pure culture; lane 2, *M. restricta* (*Mr*) amplicon incubated with *Alu*I (no restriction site recognized); lane 3, amplified target DNA from SD interscapular region skin scales identical to that of *M. restricta* (Ssc) incubated with *Alu*I (no restriction site recognized). This result was later confirmed by culture and conventional identification. The approximately 330 and 242 bp smaller amplification products conform to those of *C. albicans* and *Y.* (*Candida*) *lipolytica*, respectively, the latter also isolated in culture. No *Alu*I sites are recognized on either of these secondary products. (b) Lane 6, amplified target DNA of approximately 450 bp from trunk PV skin scales identical to that of *M. globosa* (Ssc); lane 7, *Alu*I digest of the amplification product identical to that obtained from Type and clinical pure cultures of *M. globosa* (Ssc × *Alu*I). Smearing, evident in lanes 3 and 6, was always obtained when target Ssc DNA was electrophoresed. (c) *Y. lipolytica* amplification products, *Hinf*I and *Alu*I digests from pure cultures of interscapular region SD skin scales run in 3.5% MetaPhor agarose (FMC, Rockland, ME, USA). Lanes 9 and 10, amplification products of *Y. lipolytica* (*Yl)* DNA from pure cultures; lane 11, *Hinf*I digest (*Y l* × *HinfI).* The approximately 42 bp fragment was only visible in MetaPhore agarose. Lane 12, product incubated with *Alu*I (*Yl* × *Alu*I) (no *Alu*I restriction site recognized). Lanes 4, 5 and 8, molecular size marker *Msp*I digest of pBR322 DNA.

from *Malassezia* species by the amplification product and by the RFLP pattern (Figure 4a,c).

DISCUSSION

Alternative laboratory diagnosis, based on molecular identification of each *Malassezia* species by either specific probes [14] or PCR–restriction enzyme analysis [15–17] has been employed using pure cultures to assist and confirm conventional laboratory diagnosis [5] of *Malassezia*-induced [18] or -triggered [19] dermatological conditions. Hitherto, there has not been, to our knowledge, a method for identifying *Malassezia* species by amplifying target DNA extracted directly from skin scales, which could simultaneously reveal potential interactions amongst *Malassezia* or other yeast species on human skin.

The methodology illustrated here describes the direct detection of *Malassezia* and species identification after construction of a 'library' of *Hinf*I and *Alu*I RFLPs derived from the ITS 3/4 amplicons of each pure *Malassezia* species culture. The general fungal oligonucleotide pair ITS 3/4 produced distinct amplicon sizes for two out of seven *Malassezia* species, namely *M. globosa* and *M. sympodialis*, the latter being readily distinguished by the smaller amplification product as was also noted with the ITS 1/4 primers [15].

Distinction of six out of seven *Malassezia* species, *M. furfur*, *M. globosa*, *M. restricta*, *M. sympodialis*, *M. pachydermatis* and *M. slooffiae*, was achievable upon identifying polymorphisms following digestion of the ITS 3/4 amplicon by *Hinf*I and *Alu*I. *M. obtusa* was differentiated from *M. furfur* only by the *Alu*I 3/4 distinct restriction pattern. The *M. furfur* CBS 7019 *Hinf*I generated restriction pattern was common among all clinical *M. furfur* strains tested, indicating prevalence of specific base sequences among the wild-type *M. furfur* population. Similarly, all *Hinf*I and *Alu*I restriction patterns of the clinical isolates were consistent with those obtained by sequencing the amplicons of Type and Neotype strains.

As the ITS 3/4 primers define, apart from the ITS2 intervening region portions of the 5.8S and 18S genes, the *Hinf*I restriction sites on control and clinical strains alike, appear to be in the 5.8S amplified sequences. The only exceptions included the absence of a *Hinf*I recognition site on the *M. furfur* CBS 6001 ITS 3/4 amplicon and its shift in the *M. slooffiae* amplification product.

The system proposed here allows the simultaneous detection and differentiation of different *Malassezia* and *Candida* species in a single pathological specimen. The implication of *Candida* species and *Yarrowia* (*Candida*) *lipolytica* in a *Malassezia*-induced dermatological condition, may complicate the clinical picture, delay completion of laboratory tests and, in the case of *Y. lipolytica*-associated seborrheic dermatitis, could delay ketoconazole treatment when resistant strains are implicated in the condition. This distant relative of most ascomycete yeasts, which produces proteases and lipase and utilizes *n*-paraffins [20,21], has also been isolated from pediatric systemic infections and found to be refractory in vivo and in vitro to the azoles [22]. Simultaneous isolation of *Y. lipolytica* from fungal dermatitis of captive gray seals, has been reported previously [23] but the degree of interaction between the lipase producer *Y. lipolytica* and the lipophilic *Malassezia* species has not been studied in seborrheic dermatitis, reported to recur despite application of the recommended treatment, which also includes topical ketoconazole [24,25].

Although no NCCLS break-points have been set for *Y. lipolytica*, most of the tested isolates had increased itraconazole and ketoconazole MIC. The lowest MIC, for all strains, was recorded for voriconazole, which, however, has not yet been clinically evaluated against skin infections.

Trichophyton, *Microsporum* and *Candida* species DNA from skin scales of patients has been successfully extracted previously [26]. However, in order to obtain PCR products, 2 mg of pathological skin scales were used, an unrealistically large amount when collecting specimens from the majority of *Malassezia* lesions. In the present study, the sampled amount of skin scales from these lesions was poor, and it can be particularly poor when neonates in intensive care units are sampled using a cotton swab. In such cases, the DNA-based diagnostic methods are profoundly dependent on the sensitivity of the nucleic acid extraction procedure. However, it must be acknowledged that, despite the sensitivity of the extraction method, skin scales often contain a small amount of the pathogen, contaminated by debris and PCR-inhibiting components. Therefore, the final DNA extract is frequently diminished due to wasted nucleic acids during sample decontamination and extraction. In these instances, nested PCR using the ITS 1/4 and ITS 3/4 primers can be useful in the direct detection and identification of *Malassezia* species. The benefit of using general fungal primers was the co-detection of non-*Malassezia* species in patient skin scales, for which ITS PCR-RFLP patterns are already known for many emerging opportunistic fungi [11,12,27–30].

This non-culture diagnostic method applied on dermatological specimens may be used routinely to provide detection and identification of *Malassezia* species. Admittedly, the current rate of successful extractions of 45.5% from a single specimen is low. It could be increased if more skin scales were used from parasitized specimens, but this was not always feasible, especially when sampling small and scanty lesions. However, the relative sensitivity of 10 ± 5 yeast cells, as estimated using pure cultures, matches that previously reported for *Candida* species in spiked biological fluids [11,12].

The proposed extraction method requires further testing, using more skin-scale samples, so that the observed simultaneous occurrence of more than one species from a lesion [31] could be directly detected and the results evaluated in a larger patient population. Studying more samples by using the maximum attainable skin-scale biomass will define the precise limitations, absolute sensitivity and reproducibility of this method. These results indicate that the extraction method can be applied to skin scales and demonstrate the ability of the ITS3/4 PCR-RFLP method to directly detect and identify *Malassezia* species, and their shift, upon repeated DNA extractions and PCR-RFLP experiments, from the same specimen. This agreed with our observation on the replacement of sensitive by more resistant *Malassezia* species, in cultures of these specimens.

As pathogens or potential pathogens are not always alone or prevalently implicated in skin pathology, the 'broad spectrum' detection and identification boundaries of the proposed method may be of use in molecular taxonomy, epidemiological studies and in the clinical laboratory.

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