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Microcoding and flow cytometry as a high-throughput fungal identification system for *Malassezia* species

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Yeasts of the genus *Malassezia* have been associated with a variety of dermatological disorders in humans and domestic animals. With the recent recognition of new members of the genus, new questions are emerging with regard to the pathogenesis and epidemiology of the new species. As new species are recognized, a precise and comprehensive identification system is needed. Herein is described a bead suspension culture-based array that combines the specificity and reliability of nucleic acid hybridization analysis with the speed and sensitivity of the Luminex analyser. The developed 16-plex array consisted of species- and group-specific capture probes that acted as 'microcodes' for species identification. The probes, which were designed from sequence analysis in the D1/D2 region of rRNA and internal transcribed spacer (ITS) regions, were covalently bound to unique sets of fluorescent beads. Upon hybridization, the biotinylated amplicon was detected by the addition of a fluorochrome coupled to a reporter molecule. The hybridized beads were subsequently analysed by flow cytometric techniques. The developed array, which allowed the detection of species in a multiplex and high-throughput format, was accurate and fast, since it allowed precise identification of species and required less than 1 h following PCR amplification. The described protocol, which can integrate uniplex or multiplex PCR reactions, permitted the simultaneous detection of target sequences in a single reaction, and allowed single mismatch discrimination between probe and non-target sequences. The assay has the capability to be expanded to include other medically important pathogenic species in a single or multiplex array format.

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INTRODUCTION

Medically important yeasts of the genus *Malassezia* are normal inhabitants of the human skin microflora and generally occur in areas where sebaceous glands are numerous, i.e. scalp, face, back and chest (Guého *et al.*, 1996, 1998; Gupta *et al.*, 2001, 2004a; Batra *et al.*, 2005). Under certain conditions, *Malassezia* spp. may cause dermatological disorders, such as seborrheic dermatitis (SD), dandruff, pityriasis versicolor (PV), papillomatosis, atopic dermatitis (AD), psoriasis and folliculitis (Ashbee & Evans, 2002; Batra *et al.*, 2005; Crespo-Erchiga & Florencio, 2006; Gupta *et al.*, 2004a). Some predisposing factors that can contribute to the pathogenesis of the disease include corticosteroid treatment, immunodeficiency, greasy skin, high temperature and high humidity (Guého *et al.*, 1998). *Malassezia pachydermatis* and *Malassezia furfur* can cause life-threatening fungaemia and deep mycoses in immunocompromised patients and preterm neonates (Chryssanthou *et al.*, 2001; Hazen, 1995; Morrison & Weisdorf, 2000; Richet *et al.*, 1989; Rippon, 1982; Stuart & Lane, 1992). Various *Malassezia* spp. have been isolated from animal sources and are known to cause otitis externa and skin disorders (Morris *et al.*, 2005). The most common species associated with domestic animals is *M. pachydermatis*, the only species within the genus able to sustain growth without supplementation of fatty acids (Batra *et al.*, 2005; Guillot & Bond, 1999; Morris *et al.*, 2005). This species has also been associated with various septic outbreaks in humans, some of them caused by zoonotic transfer to immunocompromised patients or preterm neonates by healthcare workers who owned dogs (Boekhout

Abbreviations: AFLP, amplified fragment length polymorphism; ITS, internal transcribed spacer; MFI, median fluorescence intensity.

& Bosboom, 1994; Chang *et al.*, 1998; Chryssanthou *et al.*, 2001; van Belkum *et al.*, 1994). However, a recent epidemiological study, which focused on the role of dogs as possible vectors of transmission on human hands, reported that mechanical transfer of *M. pachydermatis* to dog owners is of low risk to health care (Morris *et al.*, 2005).

Since 1996, the genus *Malassezia* (Baillon) has undergone several revisions to include new species (Guého *et al.*, 1996; Hirai *et al.*, 2004; Simmons & Guého, 1990; Sugita *et al.*, 2002, 2003b, 2004). These revisions, which were based on classical techniques and molecular data, have provided the information to elucidate the clinical importance of the newly recognized taxa. In the present study, we describe a molecular DNA microcoding array approach that uses the Luminex xMAP platform, a specialized flow cytometer that allows simultaneous detection of *Malassezia* species in a high-throughput format. The bead suspension hybridization assay, which employs capture probes covalently bound to 5·6 μm microspheres, involves the hybridization of species-specific capture probes, which can act as short DNA barcodes or microcodes (Summerbell *et al.*, 2005), to biotin-labelled genomic targets amplified by PCR. The technology has 100 unique sets of microspheres. Each set is individually dyed with different ratios of red and infrared fluorophores. Therefore, the suspension array can detect 100 different species in a single assay. Flow-cytometric analysis employs two lasers: a red laser, which identifies the unique fluorescence of a given microsphere set; and a green laser, which quantifies the reaction based on the fluorescence intensity of the reporter fluorophore associated to the target.

The purpose of this study was to develop a specific molecular method suitable for the rapid and reliable identification of all the presently recognized *Malassezia* species, including those that are currently under taxonomic review. Based on the described technology, we developed a DNA microcoding array that will target all the species within the *Malassezia* genus. We envision that this culture-based method would be a useful diagnostic tool in both clinical and veterinary mycology.

METHODS

Strains. Clinical and environmental DNA isolates from different geographic areas were analysed. The source of isolation and donor information are described in Table 1. The strains were maintained at 30 °C on modified Leeming and Notman (LNA) medium (Theelen *et al.*, 2001).

DNA isolation and PCR reaction. PCR reactions employed either isolated DNA from cultured cells or direct detection from cells. DNA isolation from cultured cells was achieved by the cetyl trimethyl ammonium bromide (CTAB) method (O'Donnell *et al.*, 1997). Direct DNA isolation from cultured cells employed a pin-head-sized portion of a colony of *M. pachydermatis* diluted in 15 μl sterile distilled water. The culture was grown for 2–3 days in 2 % glucose/1 % peptone/0·5 % yeast extract at 25 °C. The microcentrifuge tube was vortexed, after which 4 μl cell suspension was transferred to the PCR reaction.

Amplified products from the large subunit (LSU) D1/D2 (LrRNA) region (~600 bp) were obtained using the universal forward primer F63 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and the universal reverse primer R635 (5′-GGTCCGTGTTTCAAGACG-3′). Amplification of the internal transcribed spacer (ITS) regions, which generated amplicons ranging in size from ~650 to 800 bp, employed the forward primer ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and the fungal reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The reverse primers were biotinylated at the 5′ end. Targets were amplified in microtubes using Qiagen HotStarTaq Master Mix in final volumes of 25 or 50 μl. The master mix contained 300–5 ng template DNA, 1·5 mM $MgCl_2$, 0·4 μM of forward and reverse primer pairs, 2·5 U HotStarTaq polymerase, and 200 μM each of dGTP, dCTP, dTTP and dATP. PCR reactions, which employed an MJ Research PTC 100 thermocycler, were incubated at 95 °C for 15 min to activate the polymerase. The reactions were cycled according to the following profile: 35 cycles consisting of 30 s denaturating at 95 °C, 30 s annealing at 50 °C and 30 s extension at 72 °C. A final extension step was carried out at 72 °C for 7 min.

Multiplex PCR reactions used two primer sets in a single reaction. The first primer set consisted of F63b (5′-TAGTAACGGCGAGTGAA-3′), which is located 35 bp downstream of the F63 primer, and the reverse primer R635. The second set employed the primer combination ITS5 and ITS4. To determine the optimum conditions for PCR multiplexing, several reactions were undertaken, which employed various concentrations of the primers (0·4–0·8 μM), $MgCl_2$ (1·5–2·25 mM), dNTPs (200–300 μm) and polymerase (2·5–3·75 U). The reaction conditions were similar to those described above, except for the employment of 30 s extension at 69 °C and a final elongation step at 69 °C for 9 min. In addition, the number of cycles was increased to 40. Amplification was verified by gel electrophoresis.

Phylogenetic analysis. Phylogenetic analyses were based on sequence analysis of the D1/D2 and ITS regions. Phylogenetic trees were computed with PAUP*4.0b10 using parsimony analysis and random stepwise addition.

Probe design. Probe design employed D1/D2 and ITS sequence data, which are publicly available in GenBank (Table 1). Sequences were aligned with the MEGALIGN program (DNAStar) and screened for areas that displayed sequence divergence among the species. To facilitate probe design and quality of the oligonucleotides, the candidate sequences were analysed for thermodynamic stability and secondary structures with the Oligo program (Molecular Biology Insights). The specificity of the probes was assessed with GenBank BLAST. The capture probes were synthesized with a 5′-end amino C12 modification with standard desalting purification (IDT). The amino modification facilitated the coupling of the oligonucleotide probe sequence to the carboxylated surface of the microspheres. Coupling of the probes to unique sets of 5·6 μm polystyrene carboxylated microspheres followed the carbodiimide method (Fulton *et al.*, 1997), with slight modifications (Diaz & Fell, 2004). Coupling optimization involved the adjustment of the amount of probe in the range 0·2–0·5 nmol.

Hybridization assay. The hybridization reaction, which was carried out in 96-well plates, used 3 M TMAC solution (3 M tetramethyl ammonium chloride, 50 mM Tris, pH 8, 4 mM EDTA, pH 8·0, 0·1 % sarkosyl) (Sigma). Duplicate samples containing 5 μl biotinylated PCR product were combined with 12 μl 1× Tris/EDTA buffer (pH 8) and 33 μl of each of the labelled beads diluted in 1·5× TMAC solution. The bead mixture consisted of ~5000 microspheres for each set of probes. Prior to hybridization, the samples were denatured for 5 min at 95 °C with a PTC-100 Thermocycler (MJ Research). After denaturing, the reaction mixture was incubated for 15 min at 55 °C. The beads were pelleted by centrifugation at 2250 r.p.m. for 3 min and the unbound PCR products were carefully removed. This step was followed by 5 min incubation at

Table 1. *Malassezia* strains used in this study

CBS, strains from the Centraalbureau voor Schimmelcultures; ATCC, strains from the American Type Culture Collection; GM, strains from the collection of Gillian Midgley, St Thomas's Hospital, London; JLP, strain collection of J. L. Poirot, Hopital Saint-Antoine, Paris; MA, strain collection of F. J. Cabañes, Universitat Autònoma de Barcelona; PM, Peter Mayser, Justus Liebig University, Giessen. T, type strain.

Species/isolate	Source of isolation	Location	GenBank accession no.
M. sympodialis			
CBS 7978	Skin, patient with pityriasis versicolor	UK	
CBS 7980	Healthy skin, man	UK	
CBS 7222 T	Ear of man	USA	AY743626
CBS 9970 (MA 182)	Horse, axilla	Spain	AY743620
M. dermatis			
CBS 9169 T (M9927)	Skin lesions of an adult patient	Japan	AB070361
CBS 9170 (M9929)	Skin lesions of an atopic patient	Japan	ABO70363
***M. equi* (*nomen nudum*)**			
MA 470	Horse, anus	Spain	AY743625
MA 461	Horse, anus	Spain	AY743624
CBS 9969 (MA 146)	Horse, anus	Spain	
M. nana			
CBS 9561	Cow, normal externa	Brazil	
CBS 9557 T (NSUV-1003)	Feline, otitis externa	Japan	AB075224
CBS 9559	Cow, otitis externa	Brazil	
CBS 9561 (MNPP)	Cow, normal externa	Brazil	
CBS 9971 (MA 262)	Cat, otitis externa	Spain	
CBS 9972 (MA 314)	Cat, otitis externa	Spain	
MA 424	Cat, ear	Spain	
MA 419	Dog, otitis externa	Spain	
MA 237	Cat, ear	Spain	
MA 231	Cat, ear	Spain	AY743609
M. restricta			
CBS 7877 T	Healthy skin of man	UK	AY743607
M. globosa			
CBS 7874	Scalp of man with dandruff	UK	AY387229
CBS 7966 T	Skin, patient with pityriasis versicolor	UK	AY743604
M. pachydermatis			
CBS 4165	Ear of dog	Germany	
CBS 1879 T	Ear of dog with otitis externa	Sweden	AY743605
CBS 1885	Ear of dog with otitis externa	Sweden	AY387237
CBS 1884	Ear of dog with otitis externa	Sweden	
M. slooffiae			
CBS 7875	Scalp of man with dandruff	UK	
CBS 7972	Skin of patient with pityriasis versicolor	France	AY387247
CBS 7975	Snout of sheep	France	AY387245
M. furfur			
GM 420	Unknown	UK	
CBS 1878 T	Dandruff, man	Unknown	AY743602
CBS 9369	Scalp of patient	Canada	
CBS 7710	Skin of man	The Netherlands	AY387213
PM 316	Throat of neonate	Germany	
JLPK23	Catheter, blood	France	AY387217
CBS 9364	Forehead of a patient	Canada	
CBS 9968 (MA 73)	Sheep, groin	Spain	
CBS 7984	Healthy skin of ear of elephant	France	
M. yamatoensis			
CBS 9725 (M9985) T	Patient with seborrheic dermatitis	Japan	AB125263

Table 1. cont.

Species/isolate	Source of isolation	Location	GenBank accession no.
M. japonica			
CBS 9432 (M9967) T	Skin of healthy Japanese woman	Japan	AB105199
CBS 9431 (M9966)	Skin of healthy Japanese woman	Japan	
M. obtusa			
CBS 7876 T	Groin of man	UK	AY743629
CBS 7968	Patient with atopic dermatitis	UK	AY387138
New sp.			
CBS 9967 (MA 80)	Goat, ear	Spain	
CBS 9973 (MA 400)	Goat, ear	Spain	AY743615
MA 383	Goat, ear	Spain	AY743616
MA 333	Goat, dorsum	Spain	

55 °C. The hybridized amplicons were labelled for 5 min at 55 °C with 300 ng freshly made streptavidin-R-phycoerythrin. The samples were centrifuged and washed with 75 µl 1× TMAC. The microsphere fluorescence was analysed on the Luminex 100 analyser. Each assay was run twice. A blank and a set of positive and negative controls were included in the assay.

The multiplex capability of the assay was examined with individual sets of species-specific probes pooled into a bead mix and tested in plex formats of 1-plex, 5-plex, 10-plex and 16-plex. Each plex assay was tested with amplicons derived from single strains. The PCR conditions followed the standard protocol. Reactions were performed in duplicate and the experiment was run twice.

To test the detection of multiple targets in a single reaction, amplicons derived from a mix of genomic DNA isolates, i.e. *Malassezia globosa*, *Malassezia sympodialis* and *Malassezia slooffiae*, were tested in the hybridization assay format. The PCR reactions for detection of multiple targets were carried out as uniplex PCR reactions (F63/R635 or ITS5/ITS4) in the reaction mix or as multiplex PCR reactions with the primer sets F63b/R635 and ITS5/ITS4 in a single reaction. Uniplex reactions used the standard PCR conditions, whereas multiplex PCR reactions followed a modified protocol (40 cycles, 30 s extension at 69 °C, and a final elongation step at 69 °C for 9 min). Five or 15 µl of amplicon was used in the hybridization assay. Experiments were performed twice.

Microsphere data analysis. The data were acquired using a Luminex xMAP flow cytometer, which was interfaced to a PC and a high-speed digital signal processor. Individual sets of microspheres were analysed by a dual laser system. The red laser (636 nm) allowed classification of the microspheres into sets according to its spectral address, which is based on the simultaneous gating of red and infrared fluorescence, and is represented by the detectors FI 2: infrared fluorescence, and FI 3: red fluorescence. The green laser (532 nm), which is represented by the detector FI 1: orange fluorescence, allowed quantification of the capture probe reaction on the surface of each microsphere set. For each probe, 100 microspheres were counted, and the median fluorescence intensity (MFI) value was calculated and reported in the median mode of the acquisition detail tab of the Luminex IS100 software. This value represents the median signal intensity measured per bead set, which records a direct statistical mean of each bead analysed in a sample run. The signal to background ratio represents the MFI signals of positive controls versus the background fluorescence of samples containing all components except the amplicon target. A positive signal corresponds to a signal which is higher than twice the background level, after the background has been subtracted.

RESULTS

Probe design and specificity

Forty-eight isolates, which represented all the currently known species within the genus *Malassezia*, were studied. The location, source of isolation and donor information are listed in Table 1. GenBank accession numbers are provided for reference sequences used to generate the D1/D2 phylogenetic tree (Fig. 1).

All probes were designed from sequence analysis of the D1/D2 region, except for the probes targeting the species *M. sympodialis*, *M. pachydermatis*, *Malassezia equi* (not yet formally described) and the mini-cluster probe for *Malassezia dermatis*-new sp., which were derived from sequence analysis of the ITS region. The probe sequences and target species are given in Table 2. The selected probe sequences had a minimum G+C content of 40 %, a Tm higher than 50 °C, and two or more centrally located mismatches between the target and the non-target sequences. Most of the probes were of uniform length (21-mer), with the exception of Pequi, Pmalass, Pderm-newsp, Pnana and Pfurf-yam-jap-obt(b), which were designed as 20- and 23-mers to avoid unstable thermodynamics. All the probes were designed to perfectly match their target sequences. An exception to this rule was the cluster probe Psymp-derm-equi, which was designed as a degenerate probe to avoid unstable ΔG. This probe sequence displayed a single mismatch (located in the first base-pair position of the 5′ end) from the target sequence, *M. sympodialis* (Table 2).

Validation of the probe sequences was undertaken with the type and non-type strains listed in Table 1. A D1/D2 phylogenetic tree comprising 12 species-specific and four hierarchical cluster-specific probes was developed for the identification of *Malassezia* species (Fig. 1). The hierarchical approach, which used broad-specificity probes to target multiple species within a clade, was established to increase the confidence of the assay, as it provided a level of redundancy (Fig. 1). In addition, new emergent pathogenic species can be unveiled. Using this strategy, we

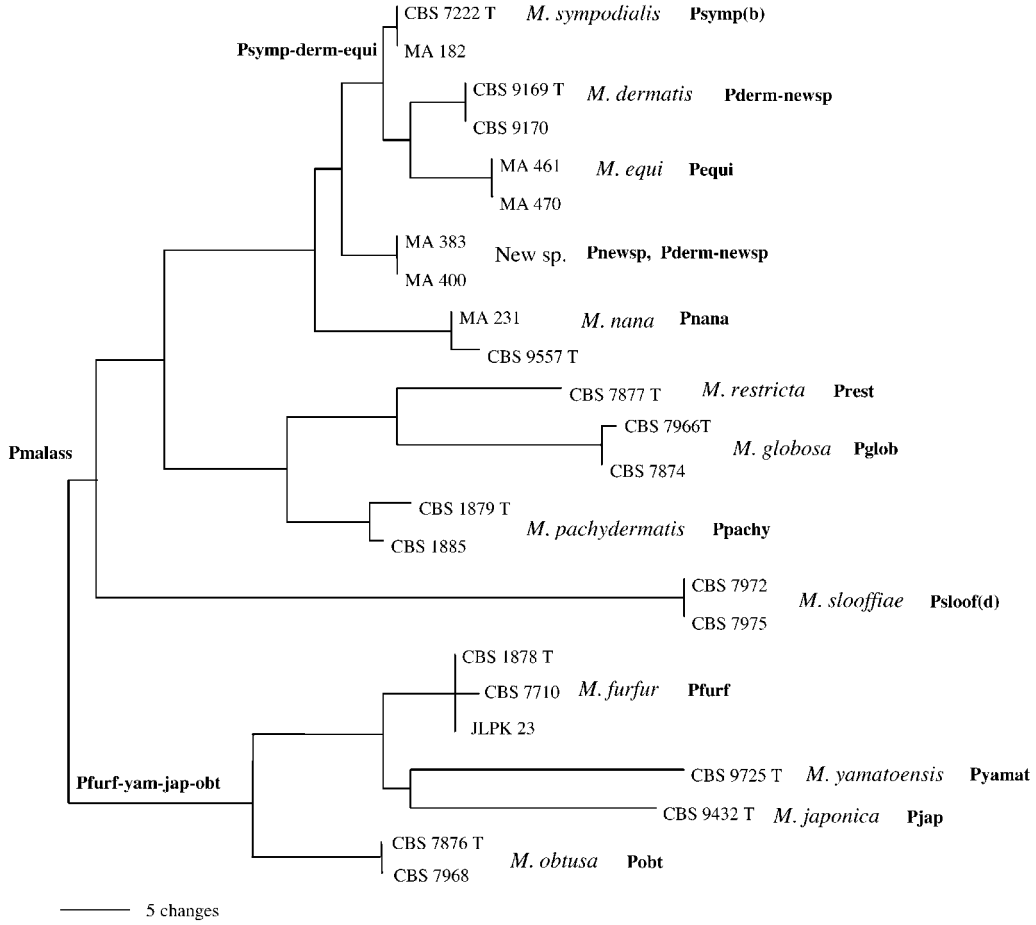

Fig. 1. Molecular phylogenetic tree of selected members of the genus *Malassezia*. The tree was generated from sequences of the D1/D2 LSU rRNA gene. Sequence data were retrieved from GenBank. The phylogenetic tree was constructed employing parsimony analysis/PAUP*4.0b 10. The developed probes are depicted in bold type.

Table 2. List of probe sequences used for *Malassezia* species- and group-specific identification

Probe	rRNA region	Target	DNA sequence (5′→3′)	Amount (nmol)
Pfurf	D1/D2	*M. furfur*	GAAGCATAGTCGGAATGTGGC	0·3
Pnana	D1/D2	*M. nana*	GTGGCACTTCGTGTGTGTTAAG	0·2
Pyamat	D1/D2	*M. yamatoensis*	TTTTGCCGAGTGTATTTCCTG	0·2
Pobt	D1/D2	*M. obtusa*	AGAAGCGTAGAAGGAATGTGG	0·2
Psymp(b)	ITS	*M. sympodialis*	AGGGAAGAGTGGCTAAGCGAC	0·3
Pderm-newsp	ITS	*M. dermatis*-new sp.	AGGGAAGGGCGGCTGAGCGA	0·3
Pequi	ITS	*M. equi*	GGGACGGGTGGCTGGGCGAC	0·4
Pglob	D1/D2	*M. globosa*	AGAAGCGCATAGGGAATGTAG	0·4
Psymp-derm-equi	D1/D2	*M. sympodialis-dermatis-equi*	GTCGCCGGAGAAGGGTGGGAG	0·3
Prest	D1/D2	*M. restricta*	TGTTATAGCCCTGTGCAGGAT	0·2
Pjap	D1/D2	*M. japonica*	AGAAGCATGGCAGGAATGTGA	0·2
Ppachy	ITS	*M. pachydermatis*	GGACTGTGTGAGTGCCTCTAG	0·2
Pfurf-yam-jap-obt(b)	D1/D2	*M. furfur-yamatoensis-japonica-obtusa*	AGCCATGCCGCTCAGGACTCAGC	0·3
Psloof(d)	D1/D2	*M. slooffiae*	CGTCTCGCTGAGGGTATTTCT	0·3
Pnewsp	D1/D2	New sp.	GATTTGGGTCACCGGAGAAGG	0·2
Pmalass	D1/D2	*Malassezia* spp.	TGGTGGTAGACTCCATCTAA	0·4

developed Pmalass, a probe that targeted all the species within the genus. In addition, two other wide taxonomic probes, i.e. Psymp-derm-equi and Pfurf-yam-jap-obt(b), were designed. As Fig. 1 illustrates, the cluster probe Psymp-derm-equi was designed to target the species *M. sympodialis*, *M. dermatis* and *Malassezia equi*, whereas Pfurf-yam-jap-obt(b) was designed to target all the species within the *M. furfur* clade, including *Malassezia yamatoensis*, *Malassezia japonica* and *Malassezia obtusa* (Fig. 1). In the same fashion, Pderm-newsp was developed to target *M. dermatis* and the new, undescribed goat isolates, whose subset we have designated 'New sp.' (Fig. 1). Conversely, several attempts were made to design a cluster probe for the identification of the species *Malassezia restricta*, *M. globosa* and *M. pachydermatis*. However, due to sequence divergences within probe designing areas, or probes that did not meet our performance criteria, we were unable to develop a cluster probe that was conserved across all the members within the clade. Technical difficulties were also encountered when developing a probe for the identification of *M. dermatis*. Therefore, identification of *M. dermatis* relied on a process of elimination with a combination of two probes, e.g. Pderm-newsp and Pnewsp (Fig. 1). This strategy, in which *M. dermatis* responded to the probe Pderm-newsp, but not to Pnewsp, allowed the successful identification of *M. dermatis*.

Fig. 2 illustrates the performance of all species-specific D1/D2 probes using the hybridization capture format assay. The probe specificity was precise, as no significant cross-reactivity was found with the non-target sequences. Similar results were obtained with cluster and ITS probes (data not shown).

To illustrate the level of specificity, each probe was tested against the complementary target amplicon (perfect match) and a selective library of amplicons representing closely and more distantly related species. The selected amplicons contained a variety of polymorphic sites representing different levels of mismatches ranging from 1 to over 6 bp. Fig. 3 illustrates the level of specificity of the assay. In this particular scenario, Pnewsp, a 21-mer probe, was tested against closely related species bearing different levels of mismatches. No significant cross-reactivity was documented for the species *Malassezia nana* and *M. dermatis*, which differed from the probe sequence by 1 or 2 bp, respectively. However, low levels of cross-reactivity were documented with *M. equi* strains (MA 146, MA 461, MA 470), which bear a mismatch identical to that found in *M. nana* strains (MA 262, CBS 9557). Nevertheless, these levels of cross-reactivity were not significant when compared to the MFI values of perfect match amplicons, which displayed fluorescent signals ~14·7-fold higher than non-target species (Fig. 3). Conversely, species that differed by two or more nucleotides from the Pnewsp probe sequence, namely *M. dermatis*, *M. sympodialis*, *M. globosa* and *M. restricta*, did not show any cross-hybridization. Our results illustrated that mismatches located in the centre or at positions 9-10-11 from the 5′ or 3′ end were discriminated using the assay conditions. A good level of specificity was also obtained with sequences displaying three consecutive mismatches at off-centred positions from the probe sequence. This was the case for Psymp-derm-equi, which did not cross-hybridize with *M. nana*, even though this species displayed off-centred base pair substitutions (C-G, A-G, C-T) at positions 4, 5 and 6 from the 3′ end of the probe sequence (data not shown).

Caution should be taken when designing a probe that can hybridize with a target that displays a centrally located insertion or deletion, as was observed with the *M. dermatis* D1/D2 probe (Fig. 4). In this case, the perceived degree of mismatch can vary with the alignment of the probe and target. For example, if the probe deletion is located at position 13 from the 5′ end, the non-target sequence, *M. slooffiae*, displays a single base pair substitution at the first position of the 5′ end, followed by two contiguous mutations (positions 11 and 12) and an insertion at position 13 (Fig. 4). This alignment can create a bulged structure consisting of three nucleotides. If the probe sequence bears a deletion at position 11, a single-base bulge may form in the target strand (Fig. 4). In both scenarios, a significant cross-reactivity was observed. Consequently, this probe sequence, which failed our

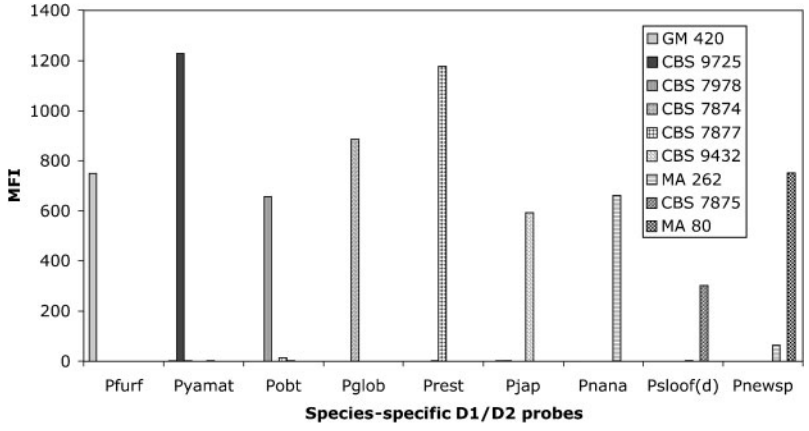

Fig. 2. Results of the performance of species-specific D1/D2 probes. The hybridization was performed at 55 °C with amplicons biotinylated at the 5′ end. All probes were tested in a multiplex format (16-plex assay). The background signal was subtracted.

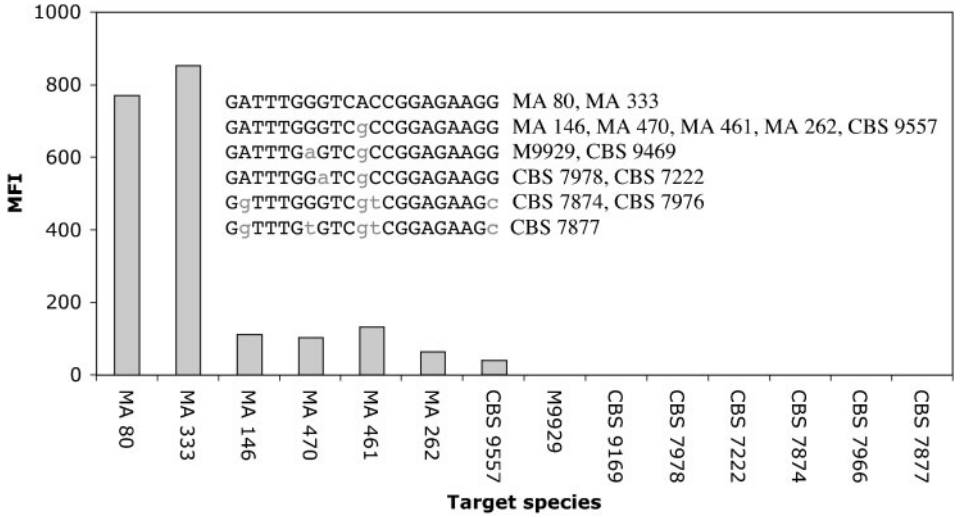

Fig. 3. Performance of the Pnewsp probe tested with other *Malassezia* spp. The probes were tested as a 16-plex format. The species depicted are: New sp. (MA 80, MA 333); *M. equi* (MA 146, MA 461, MA 470); *M. nana* (MA 262, CBS 9557); *M. dermatis* (M9929, CBS 9169); *M. sympodialis* (CBS 7978, CBS 7222); *M. globosa* (CBS 7874, CBS 7966); and *M. restricta* (CBS 7877). Nucleotide variations are shown in lower-case type.

specificity requirements, was tested but not selected for the present study.

Some variability in signal performance was observed among positive control strains when tested with their targets. For instance, Pfurf displayed signals ranging from 1040 to 750 MFI, whereas Pmalass, a probe designed to target all the species within the genus, displayed mean fluorescence signals ranging from ~364 to 220 MFI, except for strains CBS 7876, CBS 7966, CBS 9557, CBS 7984, CBS 7874, MA 146, MA 80 and CBS 4165, which displayed fluorescent signals ranging from ~195 to 106 MFI (data not shown).

Probe multiplexing

To test the multiplex capability of the suspension bead assay, different sets of probes were combined and tested with a single target amplicon per well. Overall, the MFI values for D1/D2 and ITS probes tested in various multiplex formats were similar to those observed employing a 1-plex format

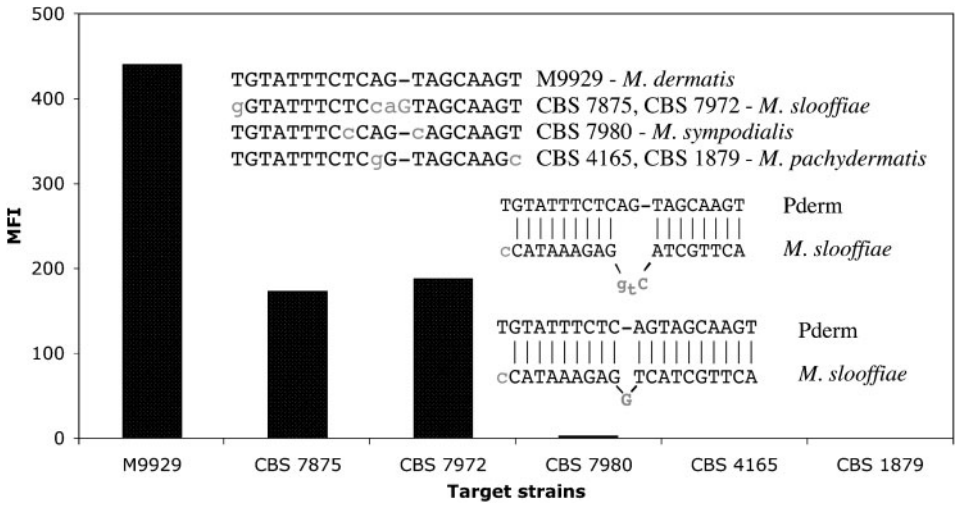

Fig. 4. Results for Pderm probe tested with other species of *Malassezia*. Sequences containing an insertion with contiguous mutations can yield false-positive results. The probes were tested as a 10-plex format. The species depicted are: *M. dermatis* (M9929); *M. slooffiae* (CBS 7875, CBS 7972); *M. sympodialis* (CBS 7980); and *M. pachydermatis* (CBS 4165, CBS 1879). Nucleotide variations are shown in lower-case type.

(non-multiplexed format). An example of the performance of various D1/D2 probes tested in multiplex formats of 1-plex (one probe), 5-plex (five probes), 10-plex (10 probes) and 16-plex (16 probes) is depicted in Fig. 5. The hybridization signals of Pglob, Prest, Pjap, Pyamat and Pnewsp were not significantly different, which demonstrates the multiplexing capability of the assay.

Direct detection from culture

The species *M. pachydermatis* was selected to explore the feasibility of omitting DNA extraction prior to PCR amplification and biotin labelling. Toward this end, we undertook direct amplification of the species with a pin-head-sized portion of a clinical culture diluted in 15 µl sterile water. Based on our results, the fluorescence signal intensity of the non-extracted cells was 8·4 % lower than the MFI values obtained from amplification reactions on isolated template DNA (data not shown). These data suggest that DNA isolation is not an essential prerequisite for successful PCR amplification and detection of *M. pachydermatis*.

Multiplex PCR reaction

Multiplex PCR reactions, which were undertaken for the simultaneous amplification of D1/D2 and ITS loci, were carried out with two primer sets in a single reaction. The PCR reaction used the same primer sets as previously described, except for F63, which was replaced by the forward primer (F63b), located 35 bp downstream of F63. Substitution of F63 was imperative, as this sequence overlaps with the reverse primer ITS4. Using the described primer sets, two amplicons of different lengths were generated and confirmed by gel electrophoresis. The multiplex PCR reactions required a certain modification/optimization to our standard PCR reactions and conditions. For instance, better amplification was documented when the PCR reactions used 2·25 mM $MgCl_2$, 300 µM dNTPs, 3·75 U polymerase and equimolar concentrations (0·6 µM) of the primer sets. In addition, better yield of products was observed when employing 40 cycles with 30 s extension at 69 °C, and a final elongation step at 69 °C for 9 min. Our results, which were obtained using a 16-plex assay format, showed that the hybridization signals and specificity of the probes Psymp(b), Pnewsp, Pnana, Pequi, Pfurf and Psloof(d) were similar to those obtained using uniplex PCR (Fig. 6). However, some species-specific probes, Pobt, Pglob, Pyamat, Prest, Pjap and Ppachy (Fig. 6), and cluster probes (data not shown), displayed reduced signal intensities when challenged with amplicons derived from multiplex PCR reactions.

Multiple target species detection (uniplex and multiplex PCR)

To determine the feasibility of the assay for the detection of multiple targets in a single reaction, a multi-template PCR reaction using uniplex and multiplex PCR assay formats was carried out with three different genomic DNAs representing the species *M. slooffiae*, *M. globosa* and *M. restricta*. The optimum conditions for the uniplex PCR reaction employed 5 ng DNA, 1·5 mM $MgCl_2$, 200 µM dNTPs, 2·5 U polymerase and 0·6 µM PCR primers. The multiplex PCR reaction used 5 ng DNA, 2·25 mM $MgCl_2$, 300 µM dNTPs, 3·75 U polymerase and equimolar concentrations (0·6 µM) of the primer sets (F63b/R635 and ITS5/ITS4). The cycling programmes were similar to those described above for uniplex and multiplex PCR assay formats, except for uniplex PCR, which used 40 cycles. Under the conditions used, our uniplex and multiplex PCR formats enabled the simultaneous amplification of the species. When the generated amplicons were challenged with their respective complementary species-specific probes, each probe reacted specifically with its target sequence. As expected, when

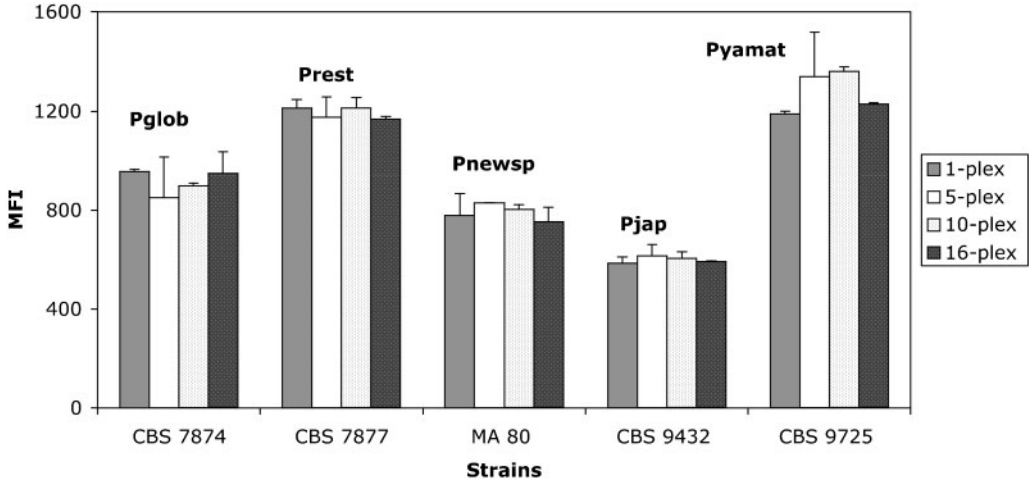

Fig. 5. Probe signal performance for Pglob, Prest, Pjap, Pyamat and Pnewsp, using different plex assay formats. Each probe was assayed individually and in a reaction mixture of five, 10 or 16. Bars, average of median + SE.

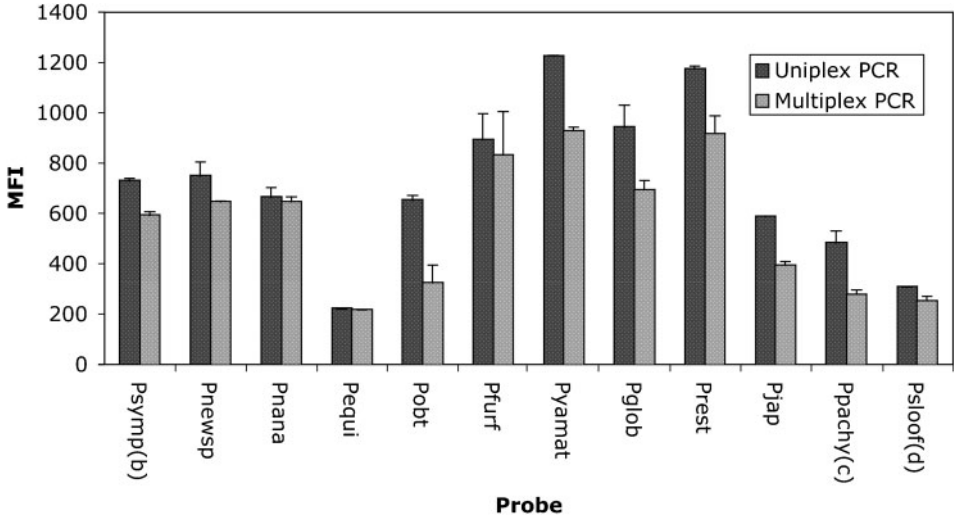

Fig. 6. Comparison of hybridization signals from targets derived from multiplex or uniplex PCR reactions. The multiplex PCR reaction used the primer sets F63b/R635 and ITS5/ITS4 in a single reaction, whereas the uniplex reaction employed either F63/R635 or ITS5/ITS4 in a reaction tube. The experiment was run twice and bars represent average of median + SE.

tested in uniplex or multiplex PCR format, the yield of amplicons derived from the multiple genomic PCR was lower than that of the single genomic PCR reaction (Fig. 7). Nevertheless, this reduction in fluorescent signal was improved by increasing the amount of amplicon in the hybridization reaction (Fig. 7). For instance, when 15 µl of the amplicon target mix was used, the hybridization signals were comparable to those obtained with single target amplicons. However, the Pglob probe displayed heightened MFI values when challenged with 5 µl of single target amplicon (CBS 7874) as opposed to 15 µl of the amplicon target mix. The same outcome was observed when either uniplex or multiplex PCR was employed (Fig. 7).

DISCUSSION

With the recent taxonomic revisions of the genus *Malassezia* (Guého *et al.*, 1996; Hirai *et al.*, 2004; Simmons & Guého,

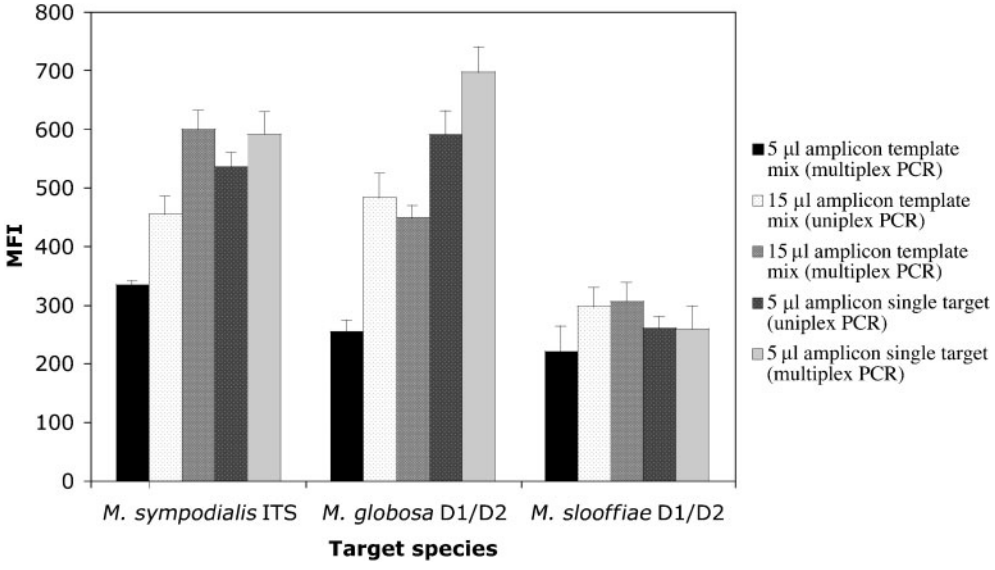

Fig. 7. Effect of various amounts of the amplicon template mix on hybridization intensities using uniplex and multiplex PCR. The PCR reactions used a genomic template mix of CBS 7978 (*M. sympodialis*), CBS 7972 (*M. slooffiae*) and CBS 7874 (*M. globosa*). Each PCR reaction used 5 ng genomic template. The signal intensities of multi-template PCR (three strains) are compared with single-target PCR (one strain). Bars, average of median + SE.

1990; Sugita *et al.*, 2002, 2003b, 2004), accurate identifications of the species are needed to obtain a better understanding of the role of each individual species in the aetiology of disease, and to facilitate adequate treatment. This can be determined based on species-specific susceptibilities to antifungal agents (Batra *et al.*, 2005).

For many decades, the genus remained limited to two species, *M. furfur* and *M. pachydermatis*. However, based on differential responses to physiological tests, morphological and molecular analyses (Guého *et al.*, 1996; Guillot & Guého, 1995; Simmons & Guého, 1990), other species have been included: *M. sympodialis* (Simmons & Guého, 1990); and *M. globosa*, *M. obtusa*, *M. slooffiae* and *M. restricta* (Guého *et al.*, 1996). This taxonomic classification is being expanded as new taxa, namely *M. dermatis* (Sugita *et al.*, 2002), *M. yamatoensis* (Sugita *et al.*, 2004), *M. japonica* (Sugita *et al.*, 2003b), *M. nana* (Hirai *et al.*, 2004) and *M. equi* (Nell *et al.*, 2002), are uncovered.

Sequence analysis of the D1/D2 regions of the 26S rRNA gene and the ITS 5·8S rRNA demonstrated that some of the newly described species, such as *M. yamatoensis*, shared molecular similarities with *M. furfur*, *M. japonica* and *M. obtusa* (Fig. 1), whereas the species *M. dermatis*, *M. equi* and *M. nana* were phylogenetically closely related to members of *M. sympodialis* (Cabañes *et al.*, 2005; Sugita *et al.*, 2004). Similar sequence analysis uncovered a subset of strains (Cluster III) within *M. sympodialis*-related strains (Cabañes *et al.*, 2005). To date, no formal species nomenclature has been assigned to this subset of strains, which we designated 'New sp.'.

Currently, the identification of *Malassezia* species relies on morphological and physiological characteristics, and this entails complicated assimilation tests involving long-chain fatty acids (C12–C24) or various Tween esters. These assimilation tests are tedious and fail to resolve some species, i.e. *M. furfur*, *M. sympodialis* and *M. slooffiae* (Crespo *et al.*, 2000; Guillot *et al.*, 1996). To differentiate these physiologically similar species, additional tests with Cremophor EL and β-glucosidase are required (Mayser *et al.*, 1997). Other approaches involve the use of molecular techniques. These techniques include mol% G+C content (Guillot & Guého, 1995), PFGE (Anthony *et al.*, 1994; Boekhout *et al.*, 1998), amplified fragment length polymorphism (AFLP) (Gupta *et al.*, 2004b; Theelen *et al.*, 2001), random amplified polymorphic DNA (RAPD) (Theelen *et al.*, 2001; Castellá *et al.*, 2005), denaturing gradient gel electrophoresis (DGGE) (Theelen *et al.*, 2001) and DNA sequence analysis (Cabañes *et al.*, 2005; Gupta *et al.*, 2004b; Sugita *et al.*, 2003a, 2005). Some of these molecular techniques have achieved success for the identification of *Malassezia* species; however, many of these approaches are not suited for clinical analysis, where a high-throughput method is needed. For instance, RFLP and AFLP analysis, which involve the complex analysis of multiple banding patterns, are lengthy and laborious to perform. In addition, interpretation of RFLP and AFLP banding patterns can be difficult when dealing with clinical specimens that contain complex communities (Gemmer *et al.*, 2002). Other techniques, such as karyotyping, can take up to 72 h for analysis after culture growth and DNA isolation (Gupta *et al.*, 2000). Similarly, PCR-restriction endonuclease analysis (PCR-REA) of rDNA requires the concurrent use of physiological tests and microscopy to clarify ambiguities between some of the species (Gupta *et al.*, 2000).

Thus, we adapted Luminex technology for the detection and identification of the medically important species within the genus *Malassezia*. A total of 16 capture probes, which included species-specific and cluster-specific probes, were developed and validated with strains that represented all currently recognized and new emergent species. The methodology illustrated here, which used a liquid suspension hybridization format, proved to be a rapid, accurate and reliable technique with the capability to be adapted to a high-throughput format.

The hybridization assay, which employed a stringent solution of 3M TMAC, proved to be specific, as we could discriminate between sequences that differed by 1 or 2 bp. However, the level of specificity depended on the location of the mismatch and on probe design. As previously described, specificity was attained when the location of the mismatch was at the mid point between the 5′ and 3′ ends (Diaz & Fell, 2004, 2005). Mismatches in the centre alter the equilibrium state of the reaction and therefore destabilize the duplex formation (Gotoh *et al.*, 1995). However, contrary to the 'empirical rules' of probe design, which postulate that mismatches at the end positions do not have a strong destabilizing effect, our study demonstrated that sequences bearing three off-centred consecutive substitutions (positions 4, 5 and 6 from the 3′ end of a 21-mer oligonucleotide) can destabilize the duplex formation, especially when one of the mismatches involves a C-T (Ikuta *et al.*, 1987). In addition, two consecutive mismatches (positions 5 and 6 from the 5′ end of a 21-mer oligonucleotide), one of which represents a C-T mismatch, can also have a similar effect (Diaz & Fell, 2005).

Precautions should be taken when designing a probe that can hybridize with a target displaying an insertion or deletion followed by one or two contiguous mutations. This was the case of the Pderm probe, which cross-reacted with *M. slooffiae* (Fig. 4). A similar effect has been reported among target sequences differing from the probe sequence by a single nucleotide deletion and a single nucleotide substitution (Hacia, 1999). Although, in theory, insertions and deletions are expected to cause a frame shift, in many cases a bulged structure is formed around the insertion or deletion area to accommodate 'perfect pairing' (von Ahsen *et al.*, 2000). Studies of the effect of duplex formation with probes or target sequences displaying small insertions or deletions have shown that bulged loop structures can dramatically influence the specificity of hybridization, as they are generally more stable and energetically favoured over formations of duplexes bearing centrally located single base

pair substitutions (Hacia, 1999; Karaman *et al.*, 2005; von Ahsen *et al.*, 2000). For instance, Karaman *et al.* (2005) have emphasized that single base deletion probes display lower hybridization specificity than single base insertion and single nucleotide substitution probes.

Several other factors can influence the specificity and efficiency of hybridization-based analysis, many of which are intrinsically related to the nucleotide sequence composition of the DNA segment being analysed. For example, highly structured nucleic acids and repetitive sequence elements, such as single nucleotide runs and direct or inverted repeats, can affect the specificity and sensitivity of the reaction, as they can lead to ambiguous binding of oligonucleotides at their target site (Diaz & Fell, 2004; Hacia, 1999; Southern *et al.*, 1999). In view of the fact that the effect of sequence context continues to be an unresolved puzzle, Lockhart *et al.* (1996) have addressed the dilemma by creating a set of heuristic rules, which involve avoidance of secondary structures and set limits to the numbers of direct repeat motifs, consecutive repeats and self-complementary regions in a probe sequence. Following these approaches we achieved a good level of specificity. In contrast, some of the designed probes did not meet all the heuristic rules, therefore a trial and error approach was necessary.

One of the advantages of the described bead suspension array is that accurate detection of the species could be achieved in a multiplex platform by mixing multiple probes in a single reaction. Our data demonstrated that slight or no compromise in fluorescent signal intensity was observed when the probes were tested in 1-plex, 5-plex, 10-plex and 16-plex formats. These results are in agreement with those of others who have tested the multiplexed capability of the technology (Diaz & Fell, 2004, 2005; Iannone *et al.*, 2000; Page & Kurtzman, 2005).

The multiplex capability of the assay was further expanded to include simultaneous detection of multiple nucleic acid loci in a single reaction. Toward this end, we tested our PCR reactions in a multiplex format to simultaneously amplify the ITS and D1/D2 regions of rRNA. The multiplex PCR reactions, which employed two primer sets in a single tube, proved to increase the high-throughput capability of the assay and reduce the amount of reagents, labour and amount of material to be analysed. It is well known that optimization of multiplex PCR reactions can prove difficult because of preferential amplification of some targets and generation of non-specific products, which can be amplified more efficiently than the desired target (Polz & Cavanaugh, 1998). With various modifications to our PCR conditions and reaction components, we were able to overcome most of these amplification biases. The multiplex PCR identified the species with 100 % specificity; however, the signal intensity of some of the tested probes was below the MFI values normally recorded in non-multiplex PCR reactions. Despite the reduction in fluorescence intensity for some probes, which can be overcome by increasing the amount of amplicon in the reaction, the majority of the probes displayed robust signals.

We also evaluated the ability of the developed array to detect several species in a multi-template PCR reaction using uniplex or multiplex PCR. To this end, we selected *M. slooffiae*, *M. globosa* and *M. restricta*, since they can coexist in human skin (Gupta *et al.*, 2001). The results, which were derived with mixtures containing equal amounts of genomic DNA, demonstrated that our method is able to detect and correctly identify multiple species in a single sample. However, to accommodate multiple strains in a single PCR reaction, and to diminish any preferential amplification of the target sequences (Polz & Cavanaugh, 1998), certain modifications to our uniplex (e.g. primer concentration, increase in PCR cycles, amplicon amount) and multiplex PCR reactions (e.g. amplicon amount) were necessary to achieve successful amplification and identification. Using the same array technology and a similar multi-template PCR format, Diaz & Fell (2005) successfully identified five different genotypic groups within *Cryptococcus neoformans* species complex.

Direct PCR with *M. pachydermatis* cells indicated that detection of this species could be done without prior DNA isolation. Similar outcomes have been reported in our laboratory with *C. neoformans* species complex (Diaz & Fell, 2005), *Trichosporon* and *Candida* spp. (data not shown). Multiple RNA copies, which in some yeasts have been reported in hundreds of copies (Prokopowich *et al.*, 2003), can explain the success of our direct PCR approach. Direct PCR amplification has also been applied to other microorganisms, such as protozoans (Tirasophon *et al.*, 1991) and bacteria (Fode-Vaughan *et al.*, 2003; Friedland *et al.*, 1994; McEwan & Wheeler, 1995).

Similar bead array systems have been used for the detection of bacterial pathogens (Dunbar *et al.*, 2003), biothreat agents (Wilson *et al.*, 2005) and clinically important yeasts, i.e. *C. neoformans* (Diaz & Fell, 2005), *Candida* spp. (Page & Kurtzman, 2005) and *Trichosporon* spp. (Diaz & Fell, 2004). Because of the sensitivity, flexibility and multiplex capability of this bead array technology, which can detect fungal DNA in the range 1–100 pg (Diaz & Fell, 2004, 2005), this high-throughput bead platform has the capability and elements necessary to be employed as a comprehensive microcode system for fungal species identification (Summerbell *et al.*, 2005). As DNA databases continue to build and become more accessible, an assemblage of barcoding systems consisting of short nucleotide segments can be established to facilitate identification of pathogenic species. This approach of DNA-based pathogenic species 'inventories' can easily be adapted to bead-based technologies, which provide a flexible and cost-effective platform that can be expanded to analyse up to 100 microcodes per well. These species inventories can be performed in areas relevant to the medical sector, food industry, national security or environmental science. Other technologies, such as 2D microarrays, can also meet barcoding principles, but their utility is hampered

by their high cost (Summerbell *et al.*, 2005). Some other advantages of the bead-based method include: faster and shorter hybridization kinetics, easier quality-control analyses, easier modification of assay formats, and robust statistical analysis (Spiro *et al.*, 2000). Overall, the flexibility of this bead suspension array, which can allow the expansion of the tests to other organisms, could become a valuable tool in clinics where accuracy and high-throughput-format assays are needed. Further studies are under way to adapt the assay to the direct detection of species with clinical material, and to evaluate the application and potential of the assay in clinical settings.

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