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Identification and typing of *Malassezia* yeasts using amplified fragment length polymorphism (AFLP^{Tm}), random amplified polymorphic DNA (RAPD) and denaturing gradient gel electrophoresis (DGGE)

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Abstract

Three molecular tools, amplified fragment length polymorphism (AFLP^{Tm}), denaturing gradient gel electrophoresis (DGGE) and random amplified polymorphic DNA (RAPD) analysis, were explored for their usefulness to identify isolates of *Malassezia* yeasts. All seven species could be separated by AFLP and DGGE. Using AFLP, four genotypes could be distinguished within *M. furfur*. AFLP genotype 4 contained only isolates from deep human sources, and ca. 80% of these isolates were from patients with systemic disease. Most of the systemic isolates belonged to a single RAPD genotype. This suggests that systemic conditions strongly select for a particular genotype. Although the clinical use of DGGE may be limited due to technical demands, it remains a powerful tool for the analysis of complex clinical samples. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: *Malassezia*; Yeast; Amplified fragment length polymorphism; Denaturing gradient gel electrophoresis; Random amplified polymorphic DNA; Identification

1. Introduction

The human and animal pathogenic yeast genus *Malassezia* Baillon belongs to the Ustilaginomycetes, which mainly comprise plant pathogens [1,2]. For a long time the genus *Malassezia* has remained limited to two species, namely the lipid-dependent *M. furfur* and the lipophilic *M. pachydermatis*. However, 25S rDNA sequences have revealed seven genetic entities [3], which currently are accepted as species [4].

The different species of *Malassezia* are known from various hosts, sites and diseases, but their pathological role has not been fully elucidated. *M. furfur* is known from healthy and diseased skin [4–6], nails [7], but also from systemic and mucosal sites, such as urine, vagina and blood [5,8–13]. *M. furfur* infections have been observed in hospitalized neonates with very low birth weight receiving intravenous lipid emulsions [9,10,13–15]. The species has also been isolated from animals [16–18]. *M. obtusa* is a rare species only known from healthy human skin. *M. slooffiae* is regularly isolated from human skin, usually in association with *M. sympodialis* or *M. globosa*. *M. sympodialis* corresponds to the former *M. furfur* serovar A [19] and is commonly isolated from healthy as well as diseased skin [20]. The species has also been isolated from healthy feline skin [21]. *M. globosa* corresponds to the original description of *Pityrosporum orbiculare* [22] and corresponds to the former serovar B of *M. furfur* [19]. It may be the most important species in pityriasis versicolor, either alone or associated with other species, such as *M. sympodialis* [23,24], but has also been isolated from a cat [21]. *M. restricta* is isolated almost exclusively from the head, including scalp, neck and face [25]. It corresponds to the former serovar C of *M. furfur* [19] and resembles

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Table 1
List of *Malassezia* isolates studied

Strain	Origin	Location
Malassezia restricta		
CBS 7877, T	skin	UK
JLP 47/1	seborrheic dermatitis	France
Malassezia sympodialis		
CBS 7222 T, EG 604	human ear	USA
CBS 7866, Leeming A5	normal skin	UK
CBS 7886	unknown	unknown
CBS 7978	pityriasis versicolor	UK
CBS 7979	skin	UK
CBS 7980	skin	UK
Sample A	scalp	unknown
Sample D	scalp	unknown
Sample E	scalp	unknown
Malassezia globosa		
CBS 7705	skin	The Netherlands
CBS 7709	seborrheic eczema	The Netherlands
CBS 7966 T, GM 35	pityriasis versicolor	UK
Sample B	scalp	unknown
Sample C	scalp	unknown
Malassezia pachydermatis		
CBS 1879, NT of *Pityrosporum pachydermatis*	dog with otitis externa	Sweden
CBS 1884	ear of dog with otitis externa	Sweden
CBS 1885	ear of dog with otitis externa	Sweden
CBS 1919	ulcerated ear of dog	Europe
CBS 6535	ear of dog	USA
CBS 6537	ear of dog	USA
CBS 6541	ear of dog	USA
CBS 6542	ear of dog	USA
L1, 1274208	blood of male neonate	USA
L2, 1277325	CSF of male neonate	USA
L3, 1286361	arterial blood of male neonate	USA
L4, 1284166	skin of male neonate	USA
L5, 1285226	skin of female neonate	USA
L6, 1288395	skin, arterial blood of female neonate	USA
L7, 1286191	skin of male neonate	USA
L8, 1273630	skin of male neonate	USA
L9, 1288083	skin of female neonate	USA
Malassezia slooffiae		
CBS 7956 T, JG 554	normal skin pig	France
CBS 7861, Leeming X1	skin of ear of 2-year-old girl	UK
CBS 7864	skin of ear of 8-month-old boy	UK
CBS 7972	pityriasis versicolor	Unknown
CBS 7973, EG 659	pityriasis versicolor	Unknown
CBS 7974	ear of man	Unknown
CBS 7975	dandruff	UK
Malassezia furfur		
CBS 7865, Leeming Z1	skin	UK
CBS 1878, NT of *Pityrosporum ovale*	dandruff	unknown
CBS 4171	ear of cow	Sweden
CBS 4172	skin of elk	Sweden
CBS 5332	infected skin	Canada
CBS 5333	skin lesion	Canada
CBS 5334	infected skin	Canada
CBS 7019, NT of *M. furfur*	pityriasis versicolor	Finland
CBS 7710	skin	The Netherlands
CBS 7860, Leeming Z2	skin of ear from neonate	UK
CBS 7867, Leeming Z4	skin of ear from neonate	UK
CBS 7969	elephant in zoo	France
CBS 7970	okapi in zoo	France
CBS 7984	healthy ear of elephant	France
CBS 7985, JG 590	wing of ostrich, zoo	France
EGC10	scalp, normal skin	France

Table 1 (continued)

Strain	Origin	Location
EL8, JG572	elephant in zoo	France
JLP K13	urine	France
JLP K15	catheter, blood	France
JLP K18	catheter, liver recipient, blood	France
JLP K23	catheter, blood	France
PM312	urine of neonate	Germany
PM314	tracheal secretion	Germany
PM315	anal swab of neonate	Germany
PM316	throat of neonate	Germany
PM317	feces of neonate	Germany
PM318	feces of neonate	Germany
PM319	nasal smear of neonate	Germany
Malassezia obtusa		
CBS 7876 T, GM 215	skin	UK
CBS 7968, GM 220	atopic dermatitis	UK
Tilletiopsis washingtonensis		
CBS 54450	OUTGROUP	

CBS = Centraalbureau voor Schimmelcultures; EG = E. Guého; GM = G. Midgley; JG = J. Guillot; JLP = J.L. Poirot; L1–L9 = P. O'Keefe and P. Kammeyer; PM = P. Mayser.

Pityrosporum ovale. The non-lipid-dependent species *M. pachydermatis* is well known as a normal cutaneous inhabitant of numerous warm-blooded animals [26,27], but it is rarely found in humans, although it has been found to cause septic outbreaks [28,29].

Understanding the clinical role of the species is greatly hampered by the difficulty involved in their isolation, cultivation and identification. Important differentiating characters have been found in amounts of guanine plus cytosine (mol%), DNA reassociation values, cell morphology, growth with different Tween non-ionic detergents as the sole lipid supplementation, the presence of catalase, temperature requirements [30], the presence of β-glucosidase revealed by the splitting of esculin and selective growth with cremophore EL [31,32]. Some attempts have been made to use molecular methods for the identification of *Malassezia* isolates, such as pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), sequencing analysis and restriction analysis of PCR amplicons of ribosomal sequences [8,9,26,33–37].

In this report we studied the application of three alternative molecular approaches, namely amplified fragment length polymorphism (AFLP^{Tm}), RAPD and denaturing gradient gel electrophoresis (DGGE). Our objectives were to differentiate the species of *Malassezia*, to understand their phylogenetic relationships, to analyze the infraspecific genetic variation, and to identify unknown isolates.

2. Materials and methods

The strains studied are listed in Table 1, and were maintained on Leeming and Notman (LNA) medium (1% w/v peptone, 0.5% w/v glucose, 0.01% w/v yeast extract, 0.4% desiccated ox bile, 0.1% v/v glycerol, 0.05% w/v glycerol monostearate, 0.05% v/v Tween 60, 1% v/v high fat cow's milk, 1.5% w/v agar in distilled water) at 30°C, or stored at −80°C [38]. The ustilaginaceous fungus *Tilletiopsis washingtonensis* CBS 54450 was used as an outgroup.

For DNA isolation cells were harvested from 4–5-day-old cultures and lyophilized. DNA was isolated according to the CTAB method [39] after grinding the cells with fine sand (40–100 mesh). The AFLP procedure was performed according to the manufacturer's AFLP Microbial Fingerprinting Protocol (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) with some modifications. Restriction ligation was performed simultaneously on 10 ng of genomic DNA, using 1 U of *Mse*I, 5 U of *Eco*RI and 3 U of T4 DNA ligase (Biolabs, Westburg, The Netherlands). The reaction took place in a total volume of 5.5 μl with 0.36 μM *Eco*RI adapter and 3.64 μM *Mse*I adapter from the AFLP Microbial Fingerprinting kit (PE Biosystems), 0.1 M NaCl, 0.91 mM Tris–HCl (pH 7.8), 0.18 mM $MgCl_2$, 0.18 mM dithiothreitol, 18 μM ATP and 91.36 μg ml^{-1} bovine serum albumin. The restriction ligation mixture was incubated for 2 h at 37°C and diluted by adding 25 μl sterile double distilled water. The first PCR reaction was performed with the two preselective primers (*Eco*RI core sequence and *Mse*I core sequence) and AFLP Amplification Core Mix from the AFLP Microbial Fingerprinting kit according to the manual, under the following conditions: 2 min at 72°C, followed by 20 cycles of 20 s at 94°C, 30 s at 56°C and 2 min at 72°C each. The PCR product was diluted by adding 25 μl sterile double distilled water. A second PCR reaction used more selective primers: *Eco*RI-A FAM and *Mse*I-G. The conditions were: 2 min at 94°C, followed by 10 cycles consisting of 20 s at 94°C, 30 s at 66°C decreasing 1°C every step of the cycle, and 2 min at 72°C, followed by 25 cycles consisting of 20 s at 94°C, 30 s at 56°C and 2 min at 72°C. The samples were prepared for acrylamide electrophoresis with the following

Table 2
A comparison of RAPD (ERIC), PFGE and AFLP patterns among selected isolates of *M. furfur*

CBS number	Other designation	PFGE [7,8]	AFLP	Primer ERIC 1	Primer ERIC 2	Primers ERIC 1+2	Overall ERIC
4171	–	II	2	H	H	G	8
4172	–	II	2	H	H	G	8
7984	JG570	I	2	H	H	G	8
5332	–	I	–	E	E	D	5
5333	–	I	4	F	F	E	6
5334	–	I′	4	D	D	C	4
6000	–	I	–	A	A	A	1
6001	–	I	–	G	G	F	7
7019	–	I′	2	A	A	A	1
7710	–	I	2	B	B	–	2
7982	EG625	I	2	C	C	C	3
–	EG4002	–	–	D	D	C	4
–	PM312	–	4	L	I	H	9C
–	PM314	–	4	L	I	H	9C
–	PM318	–	–	L	I	H	9C
–	PM319	–	4	L	I	H	9C
7983	EG658	I	1	I	I	H	9
–	JLP K5	–	4	I	I	H	9
–	JLP K13	–	4	J	I	H	9A
–	JLP K15	–	3	K	I	H	9B
–	JLP K18	–	4	K	I	H	9B
–	JLP K23	–	4	K	I	H	9B

loading mix: 1.0 μl of selective amplification product, 1.25 μl deionized formamide, 0.25 μl blue dextran in 50 mM EDTA and 0.5 μl of GeneScan-500 [ROX] size standard. After incubation for 3 min at 95°C, 1.5 μl mix was loaded and run for 3 h on a 5% polyacrylamide gel on the ABI 377 sequencer (PE Biosystems) using 1×TBE running buffer. Data were analyzed with the Bionumerics software package (version 1.01, Applied Maths, Kortrijk, Belgium), by UPGMA analysis with the Dice coefficient and the fuzzy logic option. Statistical significance of the clusters was tested with co-phenetic correlation.

RAPD analysis of a selection of isolates (see Table 2) of *M. furfur* with the primers ERIC 1 and 2 was performed as described earlier [29].

DGGE was performed according to Kowalchuk et al. [40] as follows. The first PCR reaction was carried out with primer pair NS1/NS8 (NS1 = 5′-GTAGTCATATGCTTGTCTC-3; NS8 = 5′-TCCGCAGGTTCACCTACGGA-3′), starting with a pre-dwell for 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 45 s at 50°C and 70 s at 72°C, and a post-dwell of 1 cycle for 5 min at 72°C. After staining with ethidium bromide (0.5 μg ml^{-1}), the DNA bands were excised from the gel and collected into new sterile 1.5-ml microcentrifuge tubes. The nested PCR was carried out using the NS1GC/NS2+10 primer pair (NS1GC = 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGCCAGTAGTCATATGCTTGTC-3′; NS2+10 = 5′-GAATTACCGCGGCTGCTGGC-3′) with the following PCR conditions: firstly a pre-dwell for 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 45 s at 62°C and 70 s at 70°C, and a post-dwell of 1 cycle for 5 min at 72°C.

DGGE was carried out with the Dcode Universal Mutation Detection System (Bio-Rad, Veenendaal, The Netherlands) using a 6% polyacrylamide gel with a denaturation range of 25–35%. 50 μl of the nested PCR (NS1GC/NS2+10) product was loaded into each well of the gel. Electrophoresis was performed as follows: firstly a prerun for 20 min at 10 V to reach the actual running temperature of 60°C, followed by 4 h at 150 V. The gel was stained with ethidium bromide (0.5 μg ml^{-1}), and visualized using a transilluminator at 302 nm.

3. Results

M. furfur, *M. pachydermatis*, *M. globosa*, *M. sympodialis*, *M. slooffiae*, *M. restricta* and *M. obtusa* all formed distinct clusters in our AFLP-based tree (Fig. 1). The indicated clusters were supported by high co-phenetic correlation values ranging from 89 to 100 (data not shown) indicating a high faithfulness of the dendrogram as compared with the original similarity matrix. The AFLP supports the previous suggestion that the genus consists of at least seven species. However, the phylogenetic relationships between these clusters were not well resolved in our tree, even though the AFLP banding patterns clearly differed between the species. Three lipid-dependent isolates of unknown identity, preliminarily identified as *M. furfur*, were found to belong to *M. globosa* and *M. sympodialis*. The banding pattern of *M. slooffiae* contained relatively few bands compared to those of the other species.

In *M. furfur* a distinct genetic substructure was elucidated. Four AFLP subtypes could be distinguished in this

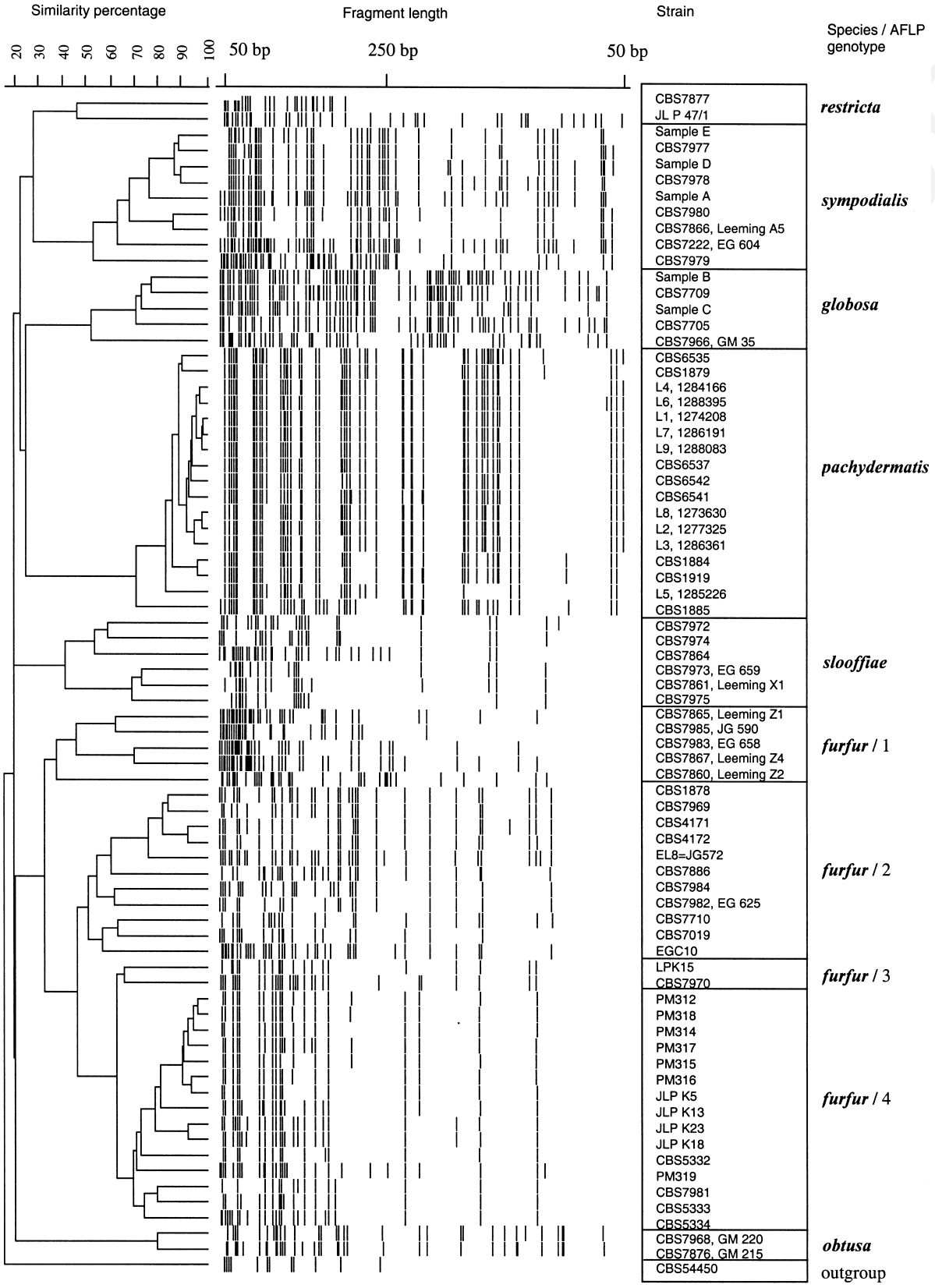

Fig. 1. AFLP patterns of *Malassezia* species. Note that in *M. furfur* four distinct genotypes occur.

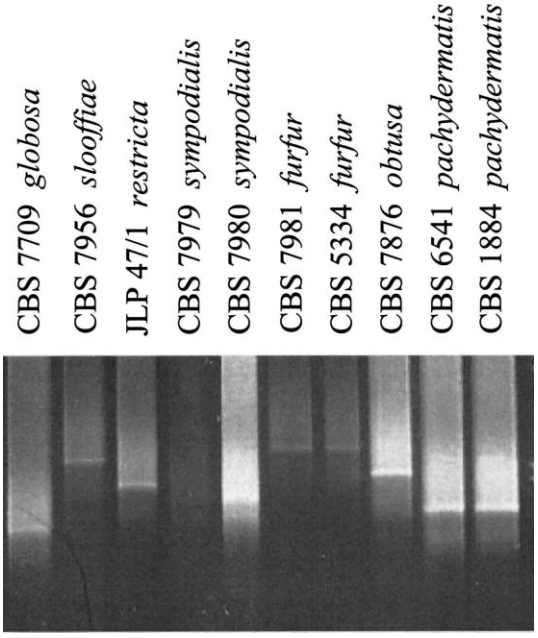

Fig. 2. DGGE patterns of a ca. 500-bp fragment of the 18S rDNA of *Malassezia* species.

species (Fig. 1). AFLP type 1 contained five isolates originating from diverse origins such as human skin, blood of a leukemic patient, and an ostrich wing. AFLP type 2 contained both the neotype strain CBS 1878 of *P. ovale* and the neotype CBS 7019 of *M. furfur*. Strains belonging to this genotype came from dandruff, human skin, pityriasis versicolor and animals such as elephant, cow and elk. The third genotype comprised only two isolates, one from a catheter and the second from an okapi. All isolates from the fourth genotype came from humans. About 80% of these were from deep or mucosal sources, such as blood, urine, tracheal secretion, anal swab, throat, nasal smear, feces, etc. The remaining ca. 20% originated from unspecified infected skin. The number of AFLP bands seen in genotype 4 is smaller than in genotypes 1–3. Karyotypes I and I′ [8] occurred in all AFLP types, whereas karyotype II was observed only in AFLP type 2. RAPD analysis of a selection of *M. furfur* isolates indicated considerable infraspecific heterogeneity as well (Table 2). All systemic isolates belonged to RAPD type 9, whereas skin isolates belonged to RAPD types 1–7, and the animal isolates to RAPD type 8. The AFLP and RAPD types were not concordant. RAPD type 9 contained AFLP types 1, 3 and 4; AFLP type 2 comprised RAPD types 1, 2, 3 and 8, and AFLP type 4 comprised RAPD types 4, 6 and 9.

M. pachydermatis formed a coherent AFLP cluster. Many of the strains studied came from dogs, but a number of isolates (L1–L9) came from neonates (skin, blood, cerebrospinal fluid) from a recent outbreak in a hospital. Apparently these hospital isolates do not genetically differ from isolates from pets, at least under the AFLP conditions used.

With DGGE, the nested PCR using primers NS1GC and NS2+10 resulted in amplicons of ca. 500 bp, which on an agarose gel could not be separated (data not shown). However, the amplicons of all species were well separated when run on a 6% acrylamide gradient gel (25–35% denaturant). No variation in electrophoretic migration was observed among different isolates of *M. furfur*, *M. pachydermatis*, and *M. sympodialis* (Fig. 2), suggesting the absence of infraspecific sequence variation within this 18S rDNA domain. Moreover, it suggests good reproducibility of the DGGE technique.

4. Discussion

4.1. AFLP^{Tm} and RAPD

AFLP is a widely used multilocus DNA typing method. It is based on the digestion of genomic DNA with two restriction enzymes, ligation of adapters to the ends of the restriction fragments, and PCR amplification using selective primers that are complementary to the restriction half sites and are elongated with one to three selective nucleotides. The method needs only small amounts of DNA, has a good reproducibility, and can be done relatively quickly [41–44].

The AFLP supports the distinction of at least seven species in *Malassezia* [4]. Therefore, the method appears useful for the identification of unknown *Malassezia* isolates. This was demonstrated by the re-identification of some unknown isolates, which were initially thought to represent *M. furfur*. A prerequisite in using AFLP for identification is that a database covering the genetic variation of the target species is available, as well as efficient software tools, such as Bionumerics (Applied Maths, Kortrijk, Belgium).

Recently molecular tools, such as PCR-RFLP of rDNA, hot-start PCR of the β-tubulin gene and of a conserved area of the lipase gene, have been explored for their usefulness in the identification of *Malassezia* yeasts [35,37]. In one approach [35], identification of isolates has been claimed to be possible within 3 days, which is comparable to the time required to perform AFLP. However, the main disadvantage of PCR-based methods is that up to three experiments may be needed to identify unknown isolates. In the second study [37] the digestion of the large subunit rDNA by three restriction enzymes is required for identification of all currently recognized species. A disadvantage of both restriction endonuclease analyses is that these techniques do not provide data on potentially clinically relevant infraspecific variation within pathogenic species.

Our discrimination of four genotypes in *M. furfur* yielded the clinically significant observation that isolates of AFLP type 4 were exclusively obtained from human sources, in 80% of these cases from deep body sites of patients with systemic diseases. However, the association

between the clinical origin and this genotype is not exclusive, as one isolate from a deep body site occurred in both AFLP types 1 and 3. These data strongly suggest that isolates with a particular genetic constitution preferentially tend to invade the human body. However, as the number of isolates studied was relatively small, future studies using more isolates are needed to confirm this hypothesis. Also, it is not clear that all mucosal isolations (e.g., from anal swab) are consistent with pathogenic invasions at these sites. In future analyses rigorous definitions of invaseness and mucosal or systemic persistence must be applied, based on reliable clinical data.

Genetic heterogeneity in *M. furfur* has been observed earlier using PFGE, RAPD analysis, sequence analysis and PCR-RFLP of the 25S rDNA [3,9,32,35,45]. Moreover, the species also presents variation in cellular morphology with globose, oval or cylindrical yeast cells [4,46].

M. pachydermatis, occurring in a wide range of warm-blooded animals [27], has been isolated from healthy human skin and from neonates in intensive care units [5,9,14,28,47,48]. The clustering of nosocomial isolates of *M. pachydermatis* together with isolates originating from pets in our AFLP analysis suggests that little genetic variation occurs within our sample. This is in agreement with the uniform karyotypes and RAPD types observed [8], but it seems to contradict the observed sequence variation in the 25S rDNA [3,27,49] and multilocus enzyme (MLEE) types [50]. This discrepancy may be due to a sampling effect, as the isolates used in our studies differed in origin from those used in the other studies. However, all studies suggested that isolates of *M. pachydermatis* from human beings belonged to a single MLEE type (type 1), sequence type (Ia), and AFLP type ([3,50], this study). This strongly suggests a correlation between genotypes within this species and the clinical significance of the isolates.

In DGGE, PCR fragments of the same size but belonging to different species and differing in their sequence can be separated, based on differences in mobility of partially denatured double-stranded DNA in a denaturing acrylamide gel [51]. This technique has found wide application in microbial ecology [40,52–54], and may be equally useful to study microbial populations involved in disease. Using our DGGE conditions, all species of *Malassezia* could be distinguished. Moreover, no infraspecific variation was detected. This renders the method useful for the identification of *Malassezia* isolates, but the clinical use may be hampered by technical demands. DGGE may be applied to the analysis of clinical samples that may comprise more species [32,46]. Such an analysis may largely contribute to our understanding of the roles of the species in different pathologies. An important condition for a successful application of DGGE in clinical samples will be the development of an efficient DNA isolation procedure, as was also suggested by Gupta et al. [35] and Guillot et al. [37].

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