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Molecular typing of *Malassezia* species with PFGE and RAPD

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The currently recognized seven species of *Malassezia* all have different karyotypes which do not vary intraspecifically, except in *M. furfur* which displayed two different karyotypes. In contrast, random amplified polymorphic DNA (RAPD) typing showed the presence of genetic variation in all species. It is concluded that karyotype analysis is useful for species identification, and RAPD typing can be used in epidemiological investigations.

Keywords *Malassezia*, PFGE, RAPD

Introduction

Species of the genus *Malassezia* are important medical yeasts. Two species have dominated the literature for a long time, namely *M. pachydermatis* (Weidman) C. W. Dodge and *M. furfur* (Robin) Baillon [1]. The genus now comprises seven species [2], which differ from each other in cellular and colony morphology, mol% G+C, serotypes, 25S ribosomal RNA/DNA sequences, requirements of long-chain fatty acids, catalase activity and temperature requirements [2]. *M. pachydermatis* is the only species not dependent on lipid supplementation, and may occur on healthy human skin and skin of other mammals [2]. *M. pachydermatis* has also been isolated from neonates receiving intravenous lipid alimentation [3–8].

The other six species are lipid-dependent. *M. furfur* may occur on healthy human and animal skin [2] and the species can cause a broad spectrum of clinical phenomena, varying from pityriasis versicolor, onychomycosis, neonatal pustulosis and sepsis in immunocompromised patients, to life-threatening invasive disease in neonates receiving lipid hyperalimentation [6,7,9–21]. *M. sympodialis* R. B. Simmons & Guého [23] is frequently isolated from healthy human skin, but also from pityriasis versicolor and folliculitis and feline skin [2,24,31]. *M. globosa* Midgley *et al*. is known from healthy human and animal skin, pityriasis versicolor and seborrhoeic dermatitis [2,31]. *M. obtusa* Midgley *et al*. has been isolated from healthy human skin and human atopic dermatitis [2,31]. *M. restricta* Guého *et al*. is known from healthy human skin (head, scalp, face and neck) and pityriasis versicolor [2,31], and *M. slooffiae* is isolated from healthy skin of humans and animals, but also from pityriasis versicolor and seborrhoeic dermatitis [2,31]. The limited data available suggest strongly that all these species may be involved in diseases of the human skin. *Pityrosporum ovale* (Bizzozero) Castellini & Chalmers (synonym *M. ovalis* (Bizzozero) Acton & Panja) and *P. orbiculare* Gordon, names commonly listed in the medical myocological literature, are currently considered conspecific with several of the lipid-dependent species [2,22]. However, it is very likely that all lipid-dependent species are still misidentified as *M. furfur* by dermatologists, epidemiologists and others, which hampers the understanding of their possible role as pathogens.

In this paper we investigated the chromosomal make-up of all seven species of *Malassezia* by pulsed field gel electrophoresis (PFGE), and analysed genetic variation within the species by random amplified polymorphic DNA (RAPD) analysis. Our results indicated that karyotypes differ between all species and that intraspecific karyotype variation is almost absent, thus suggesting that the chromosomal make-up of these species may be under evolutionary constraint. However, the mechanism of this putative process of concerted evolution is not understood. In contrast, RAPD analysis demonstrated genetic variation within all species,

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Table 1 Isolates of *Malassezia* investigated

Species	CBS number and/or other designation	Origin	Karyotype
M. furfur	5332	Infected skin, Canada	I
	5333	Skin lesion, Canada	I
	5334	Infected skin, Canada	I′
	6000	Dandruff, India	I
	6001	Pityriasis versicolor, India	I
	7019, NT of *Malassezia furfur*	Pityriasis versicolor, Finland	I′
	7043, RV 31646	?	I
	7710	Skin, The Netherlands	I
	7854	Seborrhoeic scalp, Finland	I
	7860, Leeming Z2	Skin of ear from neonate, UK	I
	7865, Leeming Z1	Skin, UK	I
	7867, Leeming Z4	Skin of ear from neonate, UK	I
	7981, EG 605	Pityriasis versicolor, France	I
	7982, EG 625	Ear, France	I
	7983, EG 658	Blood of leukaemia patient, France	I
	7984, JG 570	Healthy ear of elephant, zoo, France	I
	7985, JG 590	Wing of ostrich, zoo, France	I
	PM 312	Urine of neonate, Germany	I
	PM 315	Anal swab of neonate, Germany	I
	PM 316	Throat of neonate, Germany	I
	PM 317	Faeces of neonate, Germany	I
	PM 318	Faeces of neonate, Germany	I
	PM 319	Nasal smear of neonate, Germany	I
	JLP K5	Urine, France	I
	JLP K13	Urine, France	I
	JLP K15	Catheter, blood, France	I
	JLP K18	Catheter, liver recipient, blood, France	I
	JLP K23	Catheter, blood, France	I
	1878, NT of *Pityrosporum ovale*	Dandruff	II
	4171	Ear of cow, Sweden	II
	4172	Skin of elk, Sweden	II
M. globosa	7874, GM 29	Pityriasis capitis, UK	III
	7966T, GM 35	Pityriasis versicolor, UK	III
	7967, GM 50	Pityriasis versicolor, UK	?
	7986, GM 7	Seborrhoeic dermatitis	III
	7990	Human skin, UK	III
	7705	Skin, The Netherlands	III
	7707	Pityriasis versicolor, The Netherlands	?
	7708	Dandruff, The Netherlands	III
	7709	Seborrheic exzema, The Netherlands	III
M. sympodialis	7222T, EG 604	Human ear, USA	IV
	7859, Leeming A4	Normal skin, UK	IV
	7866, Leeming A5	Normal skin, UK	IV
	7977, EG 615	Pityriasis versicolor, France	IV
	7978, GM 314	Pityriasis versicolor, UK	IV
	7979	Skin, UK	IV
	7980	Skin, UK	IV
	8334	Domestic cat, UK	IV
M. slooffiae	7861, Leeming X1	Skin of ear of 2-year-old girl, UK	V
	7862, Leeming Y1	Skin of 1-year-old girl, UK	V
	7863, Leeming X3	Skin of ear from 4-year-old boy, UK	V
	7864, Leeming Y2	Skin of ear from 8-month-old boy, UK	V
	7875	Dandruff, UK	V
	7956T, JG 554	Skin of sheep	V

contd

Table 1 *contd*

Species	CBS number and/or other designation	Origin	Karyotype
	7971, GM 101	Human scalp	V
	7972, EG 613	Pityriasis versicolor	V
	7973, EG 659	Pityriasis versicolor	V
	7974, EG 666	Ear of man	V
	7975, JG 552	Sheep	V
M. obtusa	7876T, GM 215	Skin, UK	VI
	7968, GM 220	Atopic dermatitis, UK	VI
M. restricta	7877T	Skin, UK	VII
	JLP 44	Face of patients with seborrhoeic dermatitis, France	?
	JLP 47/1	Face of patients with seborrhoeic dermatitis, France	?
M. pachydermatis	1879, NT of *Pityrosporum pachydermatis*	Dog with otitis externa, Sweden	VIII
	1891	Ear of dog, The Netherlands	VIII
	4165	Ear of dog, Germany	VIII
	6535	Ear of dog, USA	VIII
	6541	Ear of dog, USA	VIII
	7925	Lipophilic strain from Axilla of healthy dog, UK	VIII

T, type; NT, neotype; CBS, Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands; RV, Laboratory of Mycology, Institute of Tropical Medicine, Antwerp, Belgium; EG, E. Guého; JG, J. Guillot; PM, P. Mayser; JLP, J. L. Poirot; GM, G. Midgley.

which seems to exclude clonal expansion as the sole mode of reproduction of the species. PFGE can be used for species identification, whereas RAPD analysis may be useful in epidemiological investigations.

Materials and methods

All strains investigated are listed in Table 1. Karyotype analysis and DNA isolation for RAPD analysis were performed according to Boekhout and Bosboom [7] and van Belkum *et al.* [26], respectively. For PFGE the Biorad CHEF DR-II (Biorad, Veenendaal, The Netherlands) was used with an initial pulse time of 300 s for 36 h, followed by a ramping pulse time (300–600 s) for another 36 h. Sizes of chromosomal DNA were calculated manually using commercially available-sized standards of *Saccharomyces cerevisiae* and *Pichia canadensis* (= *Hansenula wingei*) (Bio-Rad, Veenendaal, The Netherlands). For RAPD analysis 20 decamer primers (Operon Technologies, Alameda, CA, USA) were tested. The following primers gave best results: OPA 02 (TGCCGAGCTG), OPA 04 (AATCGGGCTG), OPA 05 (AGGGGTCTTG) and OPA 13 (CAGCACCCAC). Polymerase chain reaction (PCR) was performed in 200 μl reaction tubes containing 5 pmol primer, 0·1 mM dNTP, 15 mM $MgCl_2$ and 0·125 U Super/Therm (ITK Diagnostics, Uithoorn, The Netherlands) in 25 μl reaction volume. PCR used 40 cycles with the following parameters: denaturation at 94 °C for 60 s, annealing at 34 °C for 60 s and extension at 72 °C for 120 s. PFGE and RAPD products were visualized by conventional electrophoresis using 1·0% chromosomal grade agarose in 0·5 × TBE (PFGE) (Bio-Rad, Veenendaal, The Netherlands) and 1·5% agarose in 1·0 × TBA (RAPD). Ethidium bromide-stained gels were photographed on a transilluminator ($\lambda = 300$ nm). RAPD patterns were considered to belong to different types when they differed in two or more bands.

Results

Karyotyping

All currently recognized species of *Malassezia* had different karyotypes (Figs 1–7, Table 1), which in most cases lacked intraspecific variation. However, within *M. furfur* isolates, two main different karyotypes occurred (Figs 1, 2). About 90% of the isolates from diverse origin, e.g. human skin, pityriasis versicolor, blood, faeces, throat and animals, and including the neotype of *M. furfur*, CBS 7019, isolated from pityriasis versicolor (Table 1) showed type I. Judging from published figures, isolates 201, 204, 304 and 308 of Howell *et al.* [28] also

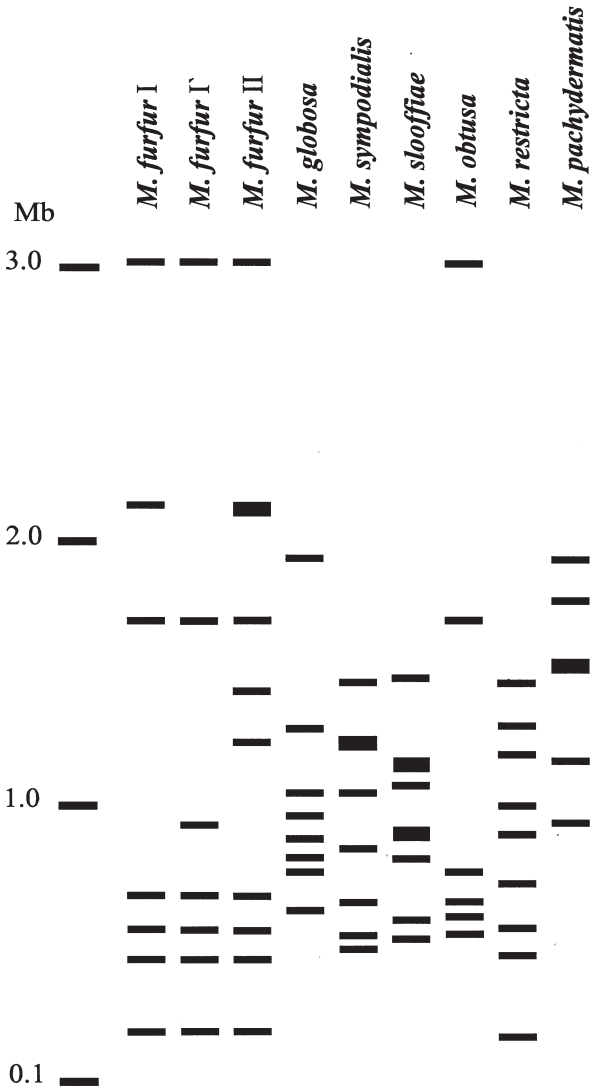

Fig. 1 Schematic representation of karyotypes of *Malassezia* species. Thick bands refer to putative double bands.

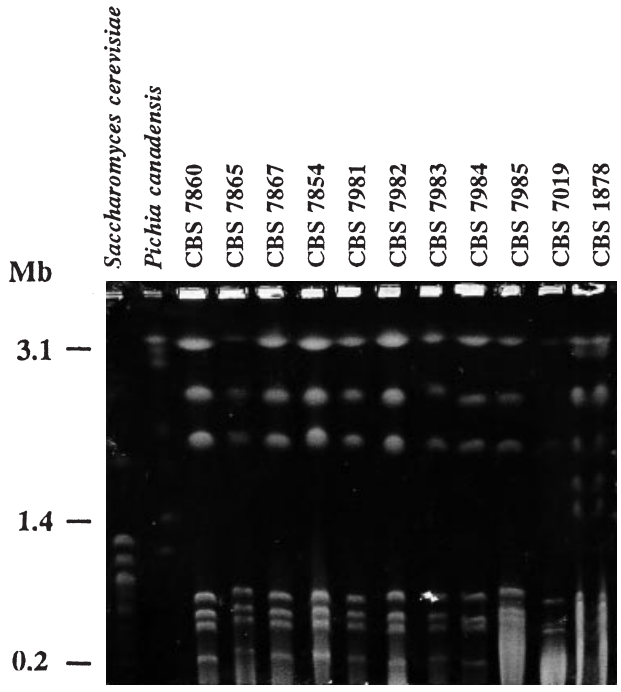

Fig. 2 Karyotypes of *Malassezia furfur.*

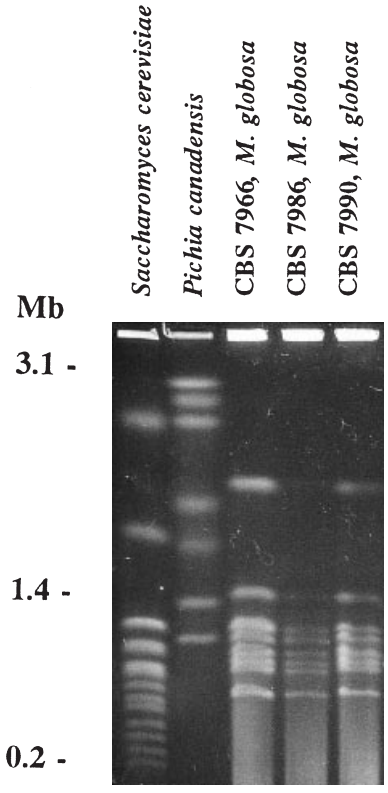

Fig. 3 Karyotypes of *Malassezia globosa.*

seem to belong here. The type I isolates contained 7–8 chromosomes, varying between *c.* 0·2 and 3·0 Mb, and resulted in an estimated total genome size of *c.* 8·5 Mb. In some of the *M. furfur* strains minor deviations occurred in the karyotypes, e.g. the replacement of the second band from top by two smaller fragments in CBS 7019 [7]. These are interpreted as subtypes (I′) of the major karyotype I. Three isolates originating from dandruff, cow ear and elk skin had identical karyotypes and possessed two additional bands of *c.* 1·5 Mb. These are referred to as type II (Table 1), and included the neotype of *P. ovale*, CBS 1878. The type II isolates had 10–11 chromosomes, varying between *c.* 0·2 and 3·0 Mb,

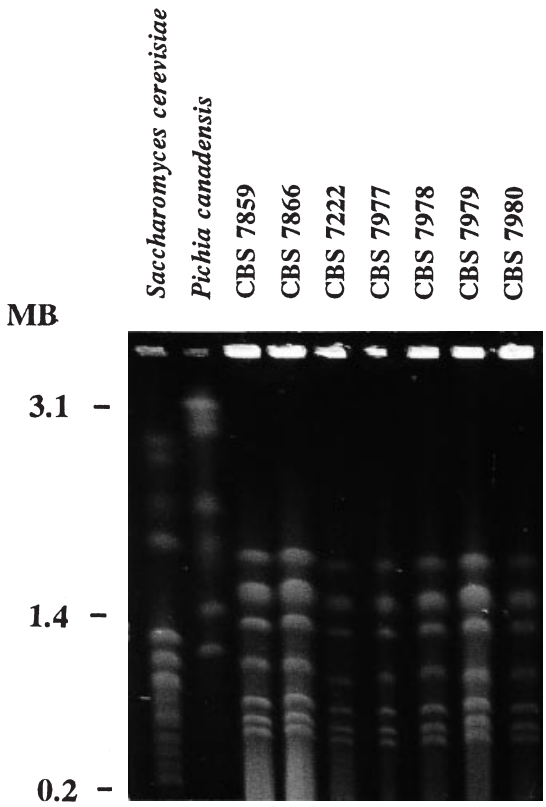

Fig. 4 Karyotypes of *Malassezia sympodialis.*

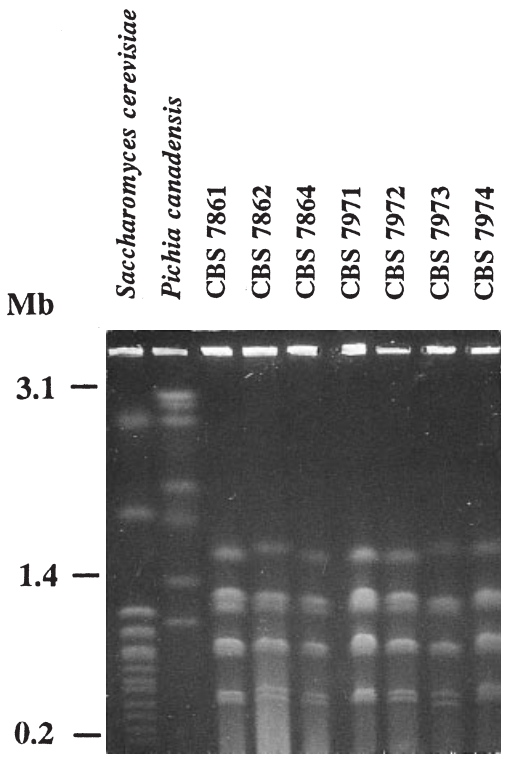

Fig. 5 Karyotypes of *Malassezia slooffiae.*

which resulted in an estimated genome size of *c*. 14 Mb, and corresponded well with our earlier observations [7]. The karyotype of *M. globosa* had 8 chromosomes varying between 0·7 and 2·0 Mb (Figs 3, 7; type III), which agreed with the third karyotype of Boekhout and Bosboom [7]. The total genome size was estimated at 8·5 Mb, and agreed well with our earlier estimate of 8·8–8·9 Mb. No intraspecific variation was observed. *M. sympodialis* contained 7 chromosomal DNA bands varying between 0·5 and 1·5 Mb (Fig. 4; type IV). They did not show intraspecific variation, matching the observations of Bond *et al.* [24]. As the second band from the top probably represented a doublet we estimated its genome size at *c*. 7·8 Mb, which is somewhat higher than the 6·4–6·6 Mb in our earlier report [7]. All isolates of *M. slooffiae* had identical karyotypes with 7 chromosomes ranging from 0·5 to 1·5 Mb (Figs 5, 7; type V). Judging from band intensity, the second and fourth bands from the top represented doublet bands, resulting in an approximate genome size of 8·6 Mb. *M. obtusa* had 6 chromosomes ranging between 0·5 and 3·0 Mb (Figs 6, 7; type VI), and an estimated total genome size of ca. 7·4 Mb. The two strains investigated were found to have identical patterns. Strain CBS 7877 of *M. restricta* had 9 chromosomes varying between 0·2 and 1·5 Mb (Fig. 7; type VII), and an estimated total genome size of *c*. 8·0 Mb. We were unable to obtain karyotypes from the remaining isolates of this species. The non-lipid dependent species *M. pachydermatis* showed five chromosomes ranging between 1·0 and 2·0 Mb, the middle one being a doublet (Fig. 1; type VIII). These results agreed well with earlier reports [7,25,29,30]. The genome size of the lipid-dependent isolate CBS 7925 [25] was estimated at ca. 7·4 Mb, thus falling into the earlier observed range of the genome size of *M. pachydermatis* of 6·7–9·3 Mb [7].

RAPD

Contrary to PFGE, RAPD analysis of *Malassezia* yeasts demonstrated genetic heterogeneity within all species. The *Malassezia* species can be distinguished by RAPD patterns (Fig. 8), but varying amounts of intraspecific genetic variation were observed using different primers. Considerable heterogeneity was observed in *M. furfur*, *M. globosa*, *M. slooffiae* and *M.*

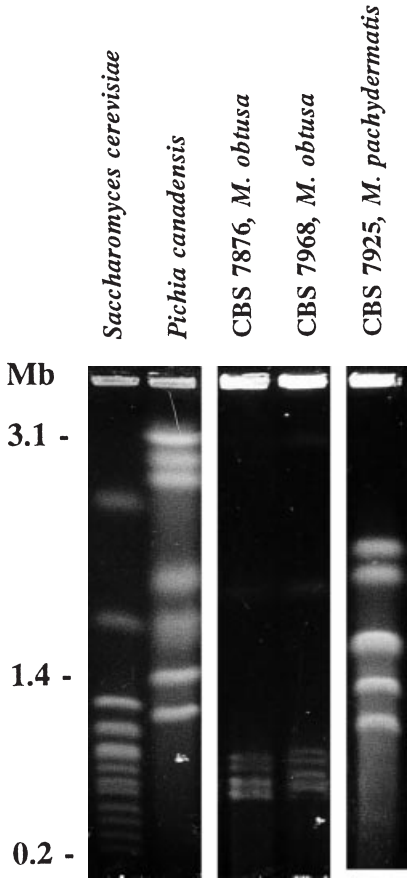

Fig. 6 Karyotypes of *Malassezia obtusa* and *M. pachydermatis.*

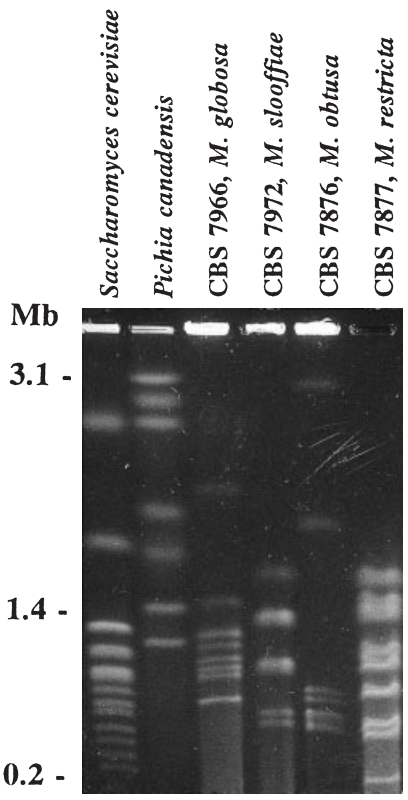

Fig. 7 Karyotypes of *Malassezia globosa*, *M. sloofiae*, *M. obtusa* and *M. restricta.*

sympodialis. Some species showed relative uniform banding patterns with certain primers, e.g. *M. sympodialis* with OPA 04 (Fig. 8b) and *M. slooffiae* with OPA 05 and 13 (Fig. 8c–d). Isolates of *M. restricta*, *M. pachydermatis* and *M. obtusa* showed uniform patterns with almost all primers investigated, but most strains could be differentiated by minor differences using all 5 OPA primers (Fig. 8a–d). In *M. furfur* no clear correlation was observed between karyotype and RAPD variation, although the karyotype II isolates showed a unique band of 860 Kb with primer OPA 05 (Fig. 8c, arrowhead). The presence of intraspecific RAPD subtypes suggest the presence of different populations, which is important from an epidemiological point of view [26]. Preliminary investigations of systemic isolates of *M. furfur* indicated that they all had karyotype I, and showed less genetic variation [32; T. Boekhout, unpublished observation)] when compared with the isolates used in this study.

Discussion

Molecular differentiation of *Malassezia* species has been achieved by PFGE, PCR typing such as RAPD analysis, restriction analysis and nucleotide sequence analysis of the large subunit of ribosomal RNA or DNA [7,27–31].

The obvious conclusion of our investigations is that all *Malassezia* species have different karyotypes, which are remarkably stable within the species. As the available data are limited for *M. obtusa* and *M. restricta* it remains to be seen whether this is also true for these species. Within *M. furfur* and *M. pachydermatis* no clear correlation is present between karyotypes and variation of large subunit (LSU) ribosomal RNA sequences. Within *M. furfur* four LSU rRNA subtypes occurred (sequence types Va–Vd), which differ by 1–2 nucleotide differences [2,31]. The majority of *M. furfur* isolates belonged to type Va and included karyotype I isolates, but also CBS 1878 with karyotype II. The neotype of *M. furfur* CBS 7019, with a slightly deviating karyotype [7], and referred to here as karyotype I′, had sequence type Vc. This apparent discrepancy between

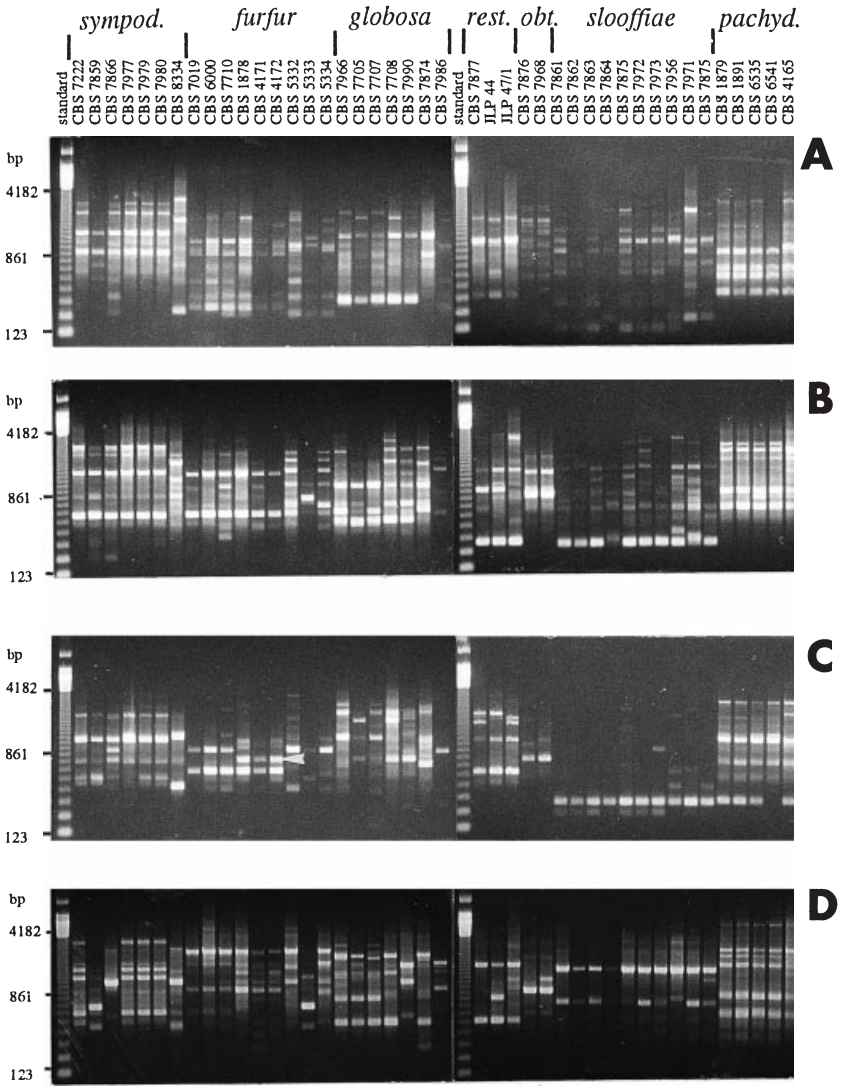

Fig. 8 RAPD patterns of species of *Malassezia.* A. Primer OPA 02; B. Primer OPA 04; C. Primer OPA 05; Primer OPA 13.

karyotypes and LSU rRNA sequences remains unexplained. Both karyotypes and LSU rRNA sequences remains unexplained. Both karyotype I and karyotype II isolates showed the same DNA base composition of ca. 66·5 mol% G+C, with DNA/DNA reassociation values higher than 85%. However, *M. furfur* in its present circumscription [2,31] is the only *Malassezia* species showing considerable morphological variation (E. Guého, unpublished observation). Despite the uniform karyotypes present within *M. pachydermatis* seven LSU rRNA sequence types have been found [31], of which the taxonomic status is not yet clear.

The lack of chromosomal length polymorphism within almost all *Malassezia* yeasts contrasts with observations on most other yeasts and moulds [33–35]. Therefore, it seems that karyotype stability of *Malassezia* yeasts is under evolutionary constraint, and we hypothesize that the genomic structure of the *Malassezia* yeasts may be different if compared with other yeasts. The occurrence of chromosomal length polymorphism (CLP) in mitotically reproducing species is usually explained by ectopic recombination through transposable elements, telomere regions and/or repeated sequences such as ribosomal DNA [34–38]. The absence of substantial CLP suggests that these DNA elements may be less common in the *Malassezia* genomes. This is supported by the relatively small genome sizes of the species [7], as well as the observation that telomeres of *M. pachydermatis* do not contain extensive poly (GT) repeats [30].

The absence of intraspecific genetic variation at the level of chromosomal DNA and its presence in the RAPD analyses is an indication for the presence of populations within the species. Therefore, PFGE may be useful to identify species of this medically important yeast genus, and RAPD analysis seems useful to screen large numbers of strains in epidemiological surveys.

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