

Molecular Differentiation of Seven *Malassezia* Species

ADITYA K. GUPTA,^{1*} YATIKA KOHLI,² AND RICHARD C. SUMMERBELL^{2,3}

Division of Dermatology, Department of Medicine, Sunnybrook Health Science Center and University of Toronto, Toronto,¹ and Department of Clinical Microbiology, The Hospital for Sick Children, Toronto,² Ontario, Canada, and Centraalbureau voor Schimmelcultures, Baarn, The Netherlands³

Received 18 August 1999/Returned for modification 1 December 1999/Accepted 2 February 2000

A system based on PCR and restriction endonuclease analysis was developed to distinguish the seven currently recognized *Malassezia* species. Seventy-eight strains, including authentic culture collection strains and routine clinical isolates, were investigated for variation in the ribosomal DNA repeat units. Two genomic regions, namely, the large subunit of the ribosomal gene and the internal transcribed spacer (ITS) region, were amplified by PCR, and products were digested with restriction endonucleases. The patterns generated were useful in identification of five out of seven *Malassezia* species. *M. sympodialis* was readily distinguishable in that its ITS region yielded a 700-bp amplified fragment, whereas the other six species yielded an 800-bp fragment. *M. globosa* and *M. restricta* were very similar in the regions studied and could be distinguished only by performing a hot start-touchdown PCR on primers for the β -tubulin gene. Primers based on the conserved areas of the *Candida cylindracea* lipase gene, which were used in an attempt to amplify *Malassezia* lipases, yielded an amplification product after annealing at 55°C only with *M. pachydermatis*. This specific amplification may facilitate the rapid identification of this organism.

Within the past decade, reviews on emerging yeast infections have repeatedly mentioned members of the *Malassezia furfur* complex as opportunistic yeasts of increasing importance (1, 18, 27, 31, 33). *Malassezia (Pityrosporum)* species are lipophilic yeasts commonly recognized as commensals of the skin of warm-blooded vertebrates that can become pathogenic under certain conditions, usually by causing the skin condition tinea (pityriasis) versicolor. Several exogenous and endogenous factors such as high temperature, high relative humidity, greasy skin, corticosteroid treatment, and immunodeficiency can influence these yeasts to become pathogenic (15).

Prior to 1990, only three *Malassezia* species were recognized. These were *M. furfur* (Robin) Baillon, *M. pachydermatis* (Weidman) C. W. Dodge, and *M. sympodialis* (Simmons and Guého). With the development of molecular techniques, new species have been segregated within *Malassezia* (16). The group of lineages formerly regarded as *M. furfur* (sensu lato [i.e., in the broad sense]) has now been divided into six species on the basis of genomic and ribosomal sequence comparisons of a large number of human and animal isolates (14). Four new taxa that have been added to *Malassezia* are *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae*.

Molecular biological studies of *Malassezia* yeasts initially consisted of determining the G+C content of chromosomal DNA (13) and direct rRNA sequencing (14, 16). Pulsed-field gel electrophoresis studies have confirmed the robustness of the new taxonomic structure of *Malassezia*, with all *Malassezia* species characterized by their individual karyotypes (5, 6, 20). Beside karyotyping, molecular differentiation of *Malassezia* species has also been attempted by PCR fingerprinting (4), restriction analysis (2), and randomly amplified polymorphic DNA analysis (6). Boekhout et al. (6) reported that although *Malassezia* species could be distinguished by randomly amplified polymorphic DNA typing, the varying amounts of heterogeneity observed within the species renders this method unreliable

for species identification. Thus, pulsed-field gel electrophoresis is the only technique that can reliably differentiate between all seven currently known *Malassezia* species. While karyotyping is very robust, its time-consuming and labor-intensive nature necessitates the development of alternative molecular methods. A rapid and reliable molecular system for identification of *Malassezia* species is needed to facilitate epidemiological and related research studies and may also be of potential utility in reference laboratories.

Comparative studies of nucleotide sequences of rRNA genes have been used extensively in molecular studies of fungi, as they provide a means for analyzing phylogenetic relationships over a wide range of taxonomic levels (7, 21, 35). Polymorphisms in the internal transcribed spacer (ITS) region and intergenic spacer of fungal ribosomal DNA repeat units, at both the inter- and intraspecific levels, have provided practical epidemiological markers for typing a range of clinically important species (8, 19, 22, 25, 26, 29). Guillot and Guého (16) had also used direct rRNA sequencing to delineate different *Malassezia* species. This method, however, cannot be used for routine analysis and diagnosis because it requires relatively large amounts of RNA and is very time-consuming. However, given that there is variability in this region, a PCR-based analysis and specific amplification of the target region would be advantageous.

In this paper we have used PCR-restriction endonuclease analysis (PCR-REA) to differentiate between the seven currently recognized *Malassezia* species. Universal fungal primers from the ITS region and specific primers designed from the published partial sequences of the large subunits (LSUs) of ribosomal genes of *Malassezia* species were used to develop a rapid and reliable PCR-restriction fragment length polymorphism (RFLP)-based system for identification of *Malassezia* species.

MATERIALS AND METHODS

Yeast strains. The sources and origins of the 78 strains investigated in this study are listed in Table 1. Of the 78 strains investigated, 64 strains were isolated from routine specimens sent to the Mycology Laboratory, Laboratories Branch, Ontario Ministry of Health, Toronto, Ontario, Canada, for fungal analysis. Among the remaining 14 strains, 6 strains were obtained from the authentic culture collections (Centraalbureau voor Schimmelcultures, Baarn, The Nether-

* Corresponding author. Mailing address: 490 Wonderland Rd. South, Suite 6, London, Ontario, Canada N6K 1L6. Phone: (519) 657-4222. Fax: (519) 657-4233. E-mail: agupta@exculink.com.

TABLE 1. Sources, origins, and multilocus genotypes of 78 strains from seven *Malassezia* species

<i>Malassezia</i> species	Strain ^a	Origin	Complex PCR-REA type ^d
<i>M. furfur</i> (n = 13)	CBS 1878, NT ^b	Dandruff	A'CFN
	JF 04	Sweden	ACFP
	GM 551	London, United Kingdom	ACFN
	97 FR-1272	Blood culture, PHLO ^c	ACFP
	97 FR-3007 ^f	Bronchial wash, PHLO	ACFN
	97 F-661 ^f	Toenail swab, PHLO	ACFN
	98 F-3617	Neck, PHLO	ACF-
	97 F-8817	Left arm, PHLO	AC-P
	98 F-5399 ^f	Trunk, PHLO	ACFP
	98 F-10017	Right index nail, PHLO	ACFN
	99 F-542	PHLO	A'CFN
	99 FR-178	PHLO	ACFN
	99 F-1436	PHLO	ACFN
<i>M. globosa</i> (n = 10)	98 F-3552	Anticubital area, PHLO	BDG-
	98 F-4888	Back, PHLO	BDGN
	98 F-6443	Body, PHLO	BDG-
	98 F-7317 ^f	Chest, PHLO	BDGN
	98 F-8304 ^f	Scalp hair, Canada	BDG-
	99 F-160	PHLO	BD-N
	YKM 48	Forehead, PHLO	B-GN
	YKM 58	Forehead, PHLO	BD-N
	YKM 45	Forehead, PHLO	BD-N
	SF7	Arm, South Africa	BDGN
<i>M. obtusa</i> (n = 3)	98 F-3529	Neck, Canada	BCGP
<i>M. obtusa</i> ^c	98 F-8316	Toenail, PHLO	ACGP
<i>M. obtusa</i> ^c	WF 7	Trunk, Hawaii	B-GN
<i>M. pachydermatis</i> (n = 3)	ATCC 14521		BCF-
	CBS 1879, NT	Dog with otitis externa, Sweden	BCFN
<i>M. pachydermatis</i> ^c	GM 420	London, United Kingdom	ACFP
<i>M. restricta</i> (n = 5)	CBS 7877, T ^c	Skin, United Kingdom	BDGP
	YKM 8	Forehead, PHLO	BDGP
	YKM 31	Scalp, PHLO	BDGP
	YKM 32	Scalp, PHLO	BDGP
	YKM 53	Scalp, PHLO	BDGP
<i>M. slooffiae</i> (n = 7)	CBS 7956, T	Healthy ear of pig	AC'GP
	JF 06	Sweden	AC'GN
	TV1 ^f	Skin, PHLO	A'-GN
	98 F-4721 ^f	Scalp, PHLO	AC'GP
	98 F-5360	Neck, PHLO	AC'GP
	98 F-6419	Groin, PHLO	A-GN
	99 F-411	PHLO	A'C'-N
<i>M. sympodialis</i> (n = 37)	CBS 7222, T	Human ear, United States	AEHN
	GM 323	London, United Kingdom	AD'-N
	97FR-2125	Skin from chest, PHLO	AEHN
	97 F-8615 ^f	Upper chest, PHLO	AEHN
	98 F-4202	Trunk, PHLO	AEHN
	98 F-4769	Back, PHLO	AEHP
	98 F-4784	Trunk, PHLO	AEHP
	98 F-4941	Abdomen, PHLO	AEG'P
	98 F-5394	Back, PHLO	AEHN
	98 F-5413	Back, PHLO	AEHN
	98 F-5763	Neck/back, PHLO	AEHP
	98 F-5850 ^f	Left breast, PHLO	AEHP
	98 F-6388	Body, PHLO	AEHN
	98 F-7582	Chest, PHLO	AEHN
	98 F-7807	PHLO	AEHN
	98 F-8046	Trunk, PHLO	AEHN
	98 F-8139	Upper back, PHLO	AEHN
	98 F-9662	Skin of scalp, PHLO	AEHN
	98 F-9846	Back, PHLO	AE-N
	98 F-9925	Trunk, PHLO	AEHN
98 F-10007	Back, PHLO	AEHN	

Continued on following page

TABLE 1—Continued

<i>Malassezia</i> species	Strain ^a	Origin	Complex PCR-REA type ^d
	98 F-10770	PHLO	AEHN
	98 F-10143	PHLO	AEHN
	98 SF-4645	CVP line swab, PHLO	AEHP
	99 F-440	PHLO	AEG'N
	99 F-869	PHLO	AD'-P
	99 F-902	PHLO	AEHN
	99 F-944	PHLO	AEHN
	99 F-946	PHLO	AE-N
	99 F-1072	PHLO	AEHN
	99 F-1181	PHLO	AEHN
	99 F-1192	PHLO	AEHN
	99 F-1407	PHLO	AEHN
	YKM 56	London, Ontario, Canada	AEHN
	YKM 73	London, Ontario, Canada	AD'HP
	SF4	Back, South Africa	AEHN

^a CBS, strains bought from the Centraalbureau voor Schimmelcultures; ATCC, strains bought from the American Type Culture Collection; GM, strains obtained as a gift from Gillian Midgley, Department of Medical Mycology, St. John's Institute of Dermatology, London, United Kingdom; JM, strains obtained as a gift from Jan Faergemann, Department of Dermatology and Venereology, Göteborg University, Göteborg, Sweden; 97/98/99 F, 97/98/99 FR, and 97/98/99 SF, strains that were sent for mycological analysis to the Medical Mycology Laboratory, Public Health Laboratories of Ontario, Etobicoke, Ontario, Canada; YKM, strains isolated by Yatika Kohli from contact plates; SF, strains isolated from specimens obtained from South Africa; WF, strains isolated from specimens obtained from Hawaii.

^b NT, neotype for *Pityrosporum ovale* (CBS 1878) or *Pityrosporum pachydermatis* (CBS 1879).

^c T, ex-type isolate.

^d The multilocus genotype represents the alleles observed for each of the strains analyzed by PCR-RFLP. The first letter designates one of the three alleles observed at the LSU locus with the restriction enzyme *Ava*I. The second letter designates one of the five alleles observed at the ITS locus with the restriction enzyme *Eco*RI. The third letter designates one of the four alleles observed at the ITS locus with restriction enzyme *Nco*I. The fourth letter designates the presence (P) or absence (N) of a fragment amplified after hot start-TD PCR with primers for the β -tubulin gene. -, missing data.

^e Some ambiguity remains regarding the correct identification of this strain (see Results).

^f Strain accessioned in the Centraalbureau voor Schimmelcultures yeast culture collection.

^g PHLO, Public Health Laboratories of Ontario, Etobicoke, Ontario, Canada.

lands, and American Type Culture Collection, Manassas, Va.), 5 strains were received as a gift from Gillian Midgley (London, United Kingdom) and Jan Faergemann (Göteborg, Sweden), and 3 strains were isolated from skin scrapings of pityriasis versicolor patients residing in Hawaii and South Africa. Before molecular analysis was conducted, identification of different *Malassezia* species among all authentic and clinical strains was performed on the basis of macro- and microscopic features and physiological characteristics as described by Guého et al. (14) and Guillot et al. (17).

DNA extraction. The fastidious growth and requirement of lipid supplements in the culture medium makes it very difficult to obtain protoplasts for DNA isolation from some strains. For successful extraction, the DNA isolation protocol was modified from the method of Sansinforiano and coworkers (34) for *Cryptococcus neoformans* and was optimized for *Malassezia*. The yeasts were grown on Leeming-Notman agar (23) for 2 to 5 days at 32°C (14). Cultures were harvested and diluted in sterile saline (0.85%) to $\sim 10^9$ CFU/ml. The cells were pelleted by centrifugation at $8,000 \times g$ for 10 min and then suspended in TE- β -mercaptoethanol buffer (280 μ l of TE buffer [100 mM Tris, 100 mM EDTA, pH 8.0], 300 μ l of deionized H₂O, 3 μ l of β -mercaptoethanol), incubated at 30°C for 45 min, and pelleted by centrifugation at $8,000 \times g$ for 10 min. They were then suspended in 1 ml of urea lysis buffer (8 M urea, 0.5 M NaCl, 20 mM Tris, 20 M EDTA, and 2% sodium dodecyl sulfate, pH 8.0), and the suspension was incubated at 37°C for at least 3 h with occasional mixing by vortexing prior to DNA extraction. The protoplasts were pelleted by centrifugation at $8,000 \times g$ for 10 min. They were resuspended in 500 to 600 μ l of lysis buffer (the same as described above but without the urea), and DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) as described by Sambrook et al. (32), followed by isopropanol precipitation and treatment with RNase at a final concentration of 10 μ g/ml. RNase treatment was followed by a chloroform-isoamyl alcohol (24:1) extraction, and the DNA was precipitated in 2 volumes of cold ethanol with high-speed centrifugation at 4°C for 15 min. The nucleic acid pellet was rinsed with cold 70% ethanol, and the air-dried pellet was suspended in 50 μ l of TE (10 mM Tris, 1 mM EDTA). Two microliters of the TE-suspended DNA was used as a template for the PCR.

This method of DNA extraction and preparation of protoplasts was also compared with extraction methods using enzymatic digestion and homogenization with glass beads for cell disruption.

Primers for PCR. Four genomic regions of *Malassezia* were amplified by PCR, namely, the LSU of the ribosomal gene, the ITS, the β -tubulin gene, and the lipase gene. The details of the primers used and the genomic regions amplified in this study are provided in Table 2.

Conventional PCR. Conventional PCR was generally performed in a 50- μ l reaction volume containing 25 μ l of *Taq* PCR master mix (the mix contains 2.5 U of *Taq* DNA polymerase, 200 μ M each deoxynucleoside triphosphate, and 1 \times PCR buffer with 1.5 mM MgCl₂) (QIAGEN Inc., Mississauga, Ontario, Canada),

0.5 μ M each primer, and 2 μ l of DNA template. DNA amplifications were carried out in a 9600 thermocycler (Perkin-Elmer, Norwalk, Conn.) programmed for initial denaturation at 95°C for 3 min followed by 40 cycles of 95, 50, and 72°C for 1 min each, with a final extension of 10 minutes at 72°C. Amplification at 55°C was also performed, but sometimes this did not yield an amplification product

TABLE 2. Primers used to screen for interspecific variation in *Malassezia* species

Locus or region	Primer set	Primer sequence (5'-3')	Product size (bp)	Reference
Large-subunit rRNA gene	26S-S ^a	GCTGAACTTAAG		16
		CATATCAT		
	26S-A ^b	TAGACGTTAGAC	642	11
		TCCTTGTT		
ITS	ITS 1	TCCGTAGGTGAA	~800/700	35
	ITS 4	CCTGCGG TCCTCCGCTTATT GATATGC		
β -Tubulin gene	Bt-2a	GGTAACCAAATC	~550	12
	Bt-2b	GGTGCTGCTTTC ACCCCTCAGTGTA GTGACCCCTGGC		
Lipase gene ^c	LIP-F	GTGTTGGCGTAC	~530	24
	LIP-R	CCGTCGTT CGAGGTCGTTGG CAAACGCA		

^a The primer designed correspond to nucleotides 1 to 20 of the partial sequence of *M. furfur* (CBS 1878; accession no. AF063214).

^b The antisense primers for the LSU rRNA gene were designed from the sequence of *Saccharomyces cerevisiae* (accession no. J01355).

^c Primers for the lipase region were designed from the sequence of the *LIP2* gene of *C. cylindracea* (accession no. X64704). A 700-bp fragment that was conserved among the five accessioned sequences of lipase genes from *C. cylindracea* was used for designing primers.

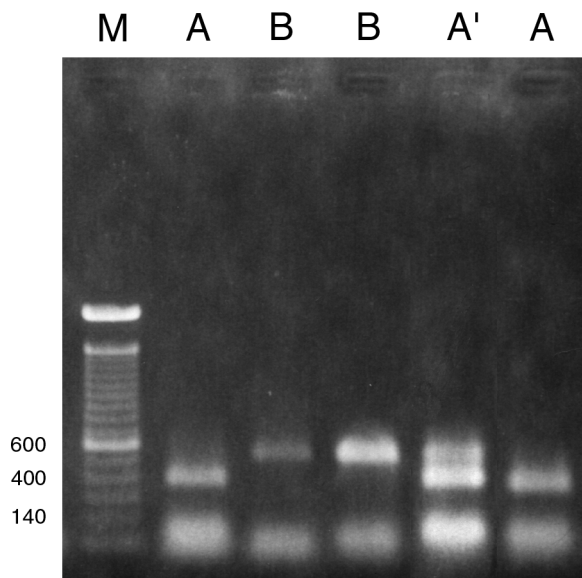


FIG. 1. Restriction patterns obtained by *Ava*I digestion of amplified product from the LSU region (primers 26S-S and 26S-A). A, B, and A', PCR-REA type; M, 100-bp ladder used as a size marker. Strains loaded in lanes from left to right, JF4 (*M. furfur*), 99F 160 (*M. globosa*), ATCC 14521 (*M. pachydermatis*), CBS 1878 (*M. furfur*), and 97F 8615 (*M. sympodialis*). A polaroid picture of the gel was scanned in the computer, and the relevant lanes were aligned together to create this image. Numbers on the left are base pairs.

(see Results). Amplified products were electrophoresed through a 0.8% agarose gel in 1× Tris-acetate-EDTA buffer, and ethidium bromide-stained gels were visualized with a UV transilluminator ($\lambda = 320$ nm).

PCR-REA. DNAs that were PCR amplified using primer sets 26S-A–26S-S and ITS 1-ITS 4 (Table 2) were subjected to further restriction endonuclease analysis. The partial sequence of LSU rRNA of *M. furfur* (GenBank accession no. AF063214) (16) reveals restriction sites for two endonucleases, *Ava*I and *Nco*I. Additionally the four-base cutters *Hae*III and *Msp*I were also used for the LSU region of *Malassezia*. To screen for interspecies variation in the ITS region of *Malassezia*, the following restriction endonucleases were used: *Ava*I, *Bam*HI, *Eco*RI, *Msp*I, *Nco*I, and *Pst*I (obtained from New England Biolabs, Mississauga, Ontario, Canada).

Hot start-TD PCR. To reduce mispriming and to increase the efficiency and specificity of amplicons obtained by conventional PCR, touchdown (TD) PCR was performed for amplification with primers for the β -tubulin gene (3). The amplification mixture (50 μ l) contained 200 μ M each deoxynucleoside triphosphate, 1× PCR buffer with 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (added as *Taq* PCR master mix) (QIAGEN Inc.), and 0.2 μ M each primer. Following a hot start, the thermocycler (9600; Perkin-Elmer) was programmed for the first cycle at 94, 67, and 72°C for 3, 1, and 1 min each. The second cycle was set at 94, 65, and 72°C for 30 s, 1 min, and 1 min each. The annealing temperature was lowered by 2°C in each of the following steps, with a final annealing temperature of 55°C. Thirty cycles were subsequently run (94°C for 30 s, 55°C for 1 min, 72°C for 1 min), ending with a final 5-min extension at 72°C.

RESULTS

DNA extraction. We compared three different methods for DNA extraction and preparation of protoplasts, using enzymatic digestion, homogenization with glass beads, and lysis in urea buffer. The last method was found to be most successful (34). The basic requirement for a good yield of DNA, however, is freshly grown (2- to 5-day-old) yeast cells. In most cases the DNA was good enough for a successful amplification, but for some strains the impurities in DNA interfered with the primers for the ITS region in PCRs. Repurification of DNA by an additional ethanol precipitation step, however, generally resulted in a positive amplification.

PCR-REA of LSU and ITS regions. PCR amplification for the LSU region with the designed primers (26S-S and 26S-A

[Table 2]) produced an amplicon of the expected size (~640 bp) for all *Malassezia* species. Restriction analysis of the amplified product was useful only with *Ava*I and not with *Nco*I. This resulted in three PCR-REA types, A, A', and B (Fig. 1; Table 3). PCR-REA of the LSU region divided the seven *Malassezia* species into two major groups. All strains of *M. sympodialis* and most strains of *M. furfur* and *M. slooffiae* showed PCR-REA type A. Only 2 out of 11 strains of *M. furfur* and 2 out of 7 strains of *M. slooffiae* showed PCR-REA type A' (the restriction pattern is a combination of those for PCR-REA types A and B [Fig. 1]). PCR-REA type B was characteristic of *M. pachydermatis*, *M. globosa*, *M. restricta*, and *M. obtusa* (Fig. 1). All of the strains were consistently amplified with these primers at an annealing temperature of 50°C. The use of a higher annealing temperature of 55°C sometimes resulted in the loss of product for some strains of *M. sympodialis*. All strains of each of the seven species gave the same restriction pattern repeatedly. A double digest of amplified product with restriction endonucleases *Hae*III and *Msp*I resulted in four different patterns (data not shown). With these enzymes, more than one restriction pattern was observed among strains of *M. furfur* and *M. globosa*. Also, the same pattern was shared by more than two *Malassezia* species (data not shown). These enzymes are being evaluated further for elucidating intraspecific variation.

PCR amplification of the ITS region with primers ITS 1 and ITS 4 (Table 2) readily distinguished *M. sympodialis* from other *Malassezia* species by its smaller amplified fragment, a 700-bp product rather than the 800-bp product produced by the others (Fig. 2A). For further species distinction with this amplicon, two restriction endonucleases proved useful. *Eco*RI divided the seven *Malassezia* species into five PCR-REA types, designated types C, C', D, D', and E (Fig. 2B; Table 3). One *Malassezia* group, showing restriction pattern type C, comprised three species, i.e., *M. furfur*, *M. pachydermatis*, and *M. obtusa* (Fig. 2B). A unique PCR-REA type, C', characterized *M. slooffiae*. PCR-REA type D comprised two species, *M. globosa* and *M. restricta* (Fig. 2B). Most strains of *M. sympodialis* showed PCR-REA type E (Fig. 2B), whereas 3 of 37 strains showed PCR-REA type D' (with a single fragment ~200 bp larger than that of type E) (Fig. 2B). *Nco*I divided the seven *Malassezia* species into four PCR-REA types, represented as F, G, G', and H (Fig. 2C; Table 3). As with *Eco*RI, *M. furfur*

TABLE 3. Molecular differentiation of *Malassezia* species by PCR-REA

Genomic region	Restriction endonuclease	PCR-REA types (<i>Malassezia</i> species ^a)
26S rRNA gene (LSU)	<i>Ava</i> I	A (<i>Mf</i> , <i>Msy</i> , <i>Msl</i>); A' (<i>Mf</i> , <i>Msl</i>); B (<i>Mg</i> , <i>Mr</i> , <i>Mo</i> , <i>Mp</i>)
TS (ITS 1-ITS 4)	<i>Eco</i> RI	C (<i>Mf</i> , <i>Mp</i> , <i>Mo</i>); C' (<i>Msl</i>); D (<i>Mg</i> , <i>Mr</i>); D' ^b , E (<i>Msy</i>)
	<i>Nco</i> I	F (<i>Mf</i> , <i>Mp</i>); G (<i>Mg</i> , <i>Mr</i> , <i>Mo</i> , <i>Msl</i>); G' ^c , H (<i>Msy</i>)
β -Tubulin gene		P (<i>Mr</i> , <i>Mf</i> , <i>Msy</i> , <i>Msl</i> , <i>Mp</i> , <i>Mo</i>) ^d ; N (<i>Mg</i> , <i>Mf</i> , <i>Msy</i> , <i>Msl</i> , <i>Mp</i>) ^e

^a *Mf*, *M. furfur*; *Msl*, *M. slooffiae*; *Msy*, *M. sympodialis*; *Mp*, *M. pachydermatis*; *Mo*, *M. obtusa*; *Mg*, *M. globosa*; *Mr*, *M. restricta*.

^b Only 3 of 37 *M. sympodialis* strains had this PCR type (see Fig. 2B).

^c Only 2 of 37 *M. sympodialis* strains had this PCR type. Restriction with *Nco*I produced two bands of 500 and 200 bp (PCR-REA type G') instead of one 350-bp band of double intensity (PCR-REA type H).

^d Positive amplification with primers for β -tubulin gene.

^e Negative amplification with primers for β -tubulin gene.

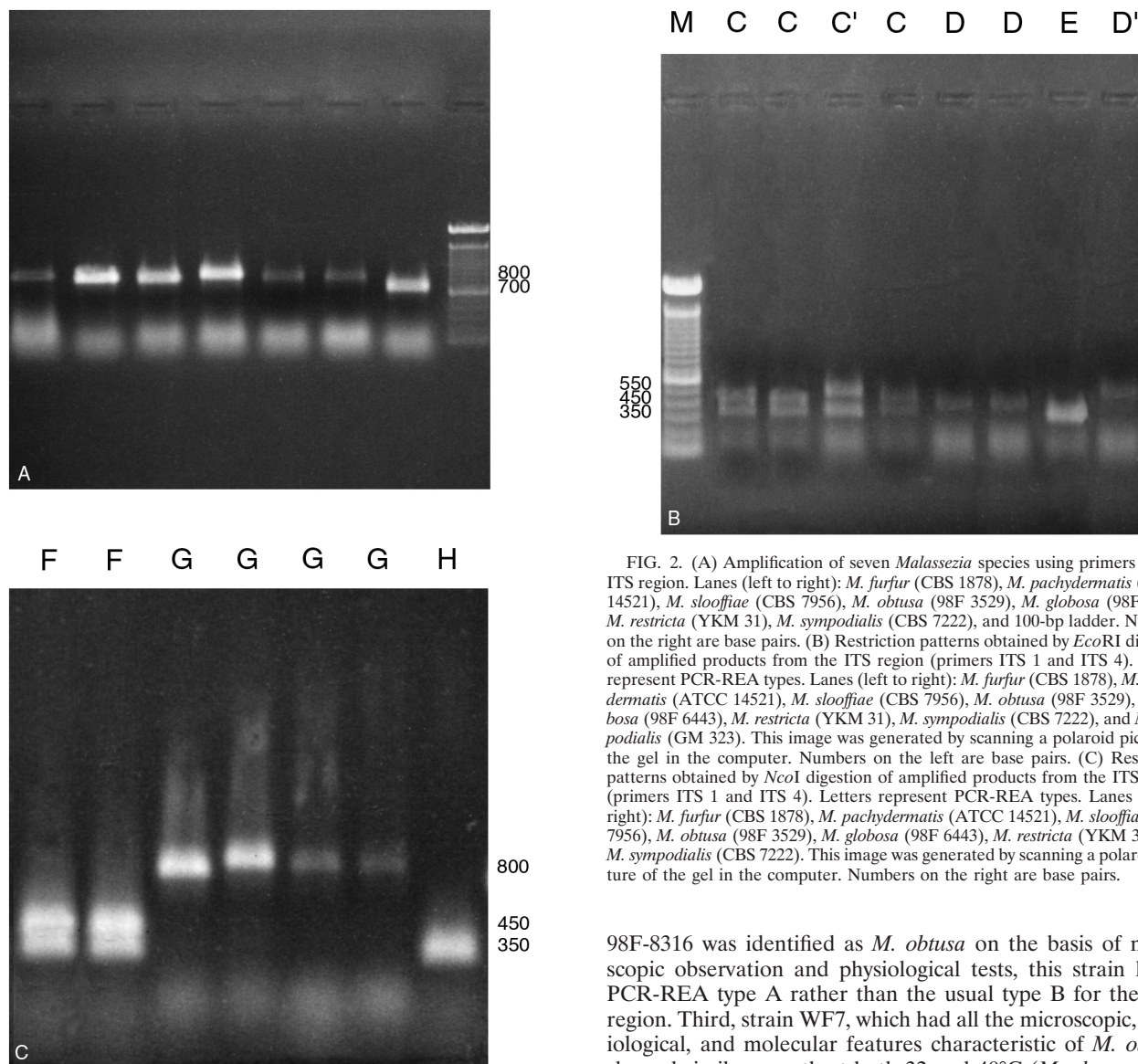


FIG. 2. (A) Amplification of seven *Malassezia* species using primers for the ITS region. Lanes (left to right): *M. furfur* (CBS 1878), *M. pachydermatis* (ATCC 14521), *M. slooffiae* (CBS 7956), *M. obtusa* (98F 3529), *M. globosa* (98F 6443), *M. restricta* (YKM 31), *M. sympodialis* (CBS 7222), and 100-bp ladder. Numbers on the right are base pairs. (B) Restriction patterns obtained by *Eco*RI digestion of amplified products from the ITS region (primers ITS 1 and ITS 4). Letters represent PCR-REA types. Lanes (left to right): *M. furfur* (CBS 1878), *M. pachydermatis* (ATCC 14521), *M. slooffiae* (CBS 7956), *M. obtusa* (98F 3529), *M. globosa* (98F 6443), *M. restricta* (YKM 31), *M. sympodialis* (CBS 7222), and *M. sympodialis* (GM 323). This image was generated by scanning a polaroid picture of the gel in the computer. Numbers on the left are base pairs. (C) Restriction patterns obtained by *Nco*I digestion of amplified products from the ITS region (primers ITS 1 and ITS 4). Letters represent PCR-REA types. Lanes (left to right): *M. furfur* (CBS 1878), *M. pachydermatis* (ATCC 14521), *M. slooffiae* (CBS 7956), *M. obtusa* (98F 3529), *M. globosa* (98F 6443), *M. restricta* (YKM 31), and *M. sympodialis* (CBS 7222). This image was generated by scanning a polaroid picture of the gel in the computer. Numbers on the right are base pairs.

and *M. pachydermatis* showed the same restriction pattern, in this case type F. *M. obtusa* and *M. slooffiae*, however, showed a different restriction pattern, type G, that was the same as for *M. globosa* and *M. restricta* (Fig. 2C). Again, *M. sympodialis* was unique, with either type G' or type H (Fig. 2C).

Five of the seven *Malassezia* species could thus be distinguished using the combination of the LSU and ITS regions. Multilocus genotypes of seven *Malassezia* species were constructed using these two regions and the commonly represented PCR-REA types (Table 4). A similarity index was calculated for all *Malassezia* species in a pairwise comparison on the basis of number of matches (Table 4).

Although in most cases the results of molecular and nonmolecular tests for the strains under investigation were in agreement, there were four ambiguous cases (Table 1). First, strain 99F-1436 was identified as *M. globosa* on the basis of microscopic examination and utilization of tween compounds (Tween 20, 40, 60, and 80) (17), but the molecular features of this strain were characteristic of *M. furfur*. Second, while strain

98F-8316 was identified as *M. obtusa* on the basis of microscopic observation and physiological tests, this strain had a PCR-REA type A rather than the usual type B for the LSU region. Third, strain WF7, which had all the microscopic, physiological, and molecular features characteristic of *M. obtusa*, showed similar growth at both 32 and 40°C (*M. obtusa* is not known to grow well at 40°C) (14). Lastly, strain GM 420, although identified as *M. pachydermatis* on the basis of physiological properties, had molecular features consistent with *M. furfur*. For this strain, however, the PCR amplification with primers for the lipase gene resulted in a product similar to that of *M. pachydermatis* strains (see below).

TD-PCR for β -tubulin gene. Initial screening by conventional PCR with primers for the β -tubulin gene (Table 2) (12), suggested that this region might be useful in differentiating *Malassezia* species (data not shown). However, consistent bands were not obtained in repeated PCRs for the same strains done under the same conditions. A hot start-TD PCR was performed for this region to determine if it would yield more consistent results. TD-PCR, by starting the annealing process at 12 to 15°C above the calculated T_m and gradually decreasing the annealing temperature, has been shown to increase the efficiency and specificity of amplicons obtained by conventional PCR (10). Primers for the β -tubulin gene proved useful in differentiating *M. globosa* and *M. restricta* by the presence or absence of an amplified fragment following a TD-PCR procedure. All strains of *M. restricta* were consistently amplified,

TABLE 4. Similarity matrix of seven *Malassezia* species on the basis of PCR-REA types of the LSU and ITS regions

<i>Malassezia</i> species	Multilocus genotype ^a	Similarity Index ^b with:					
		<i>M. pachydermatis</i>	<i>M. sympodialis</i>	<i>M. slooffiae</i>	<i>M. obtusa</i>	<i>M. globosa</i>	<i>M. restricta</i>
<i>M. furfur</i>	101000100	0.77	0.55	0.55	0.55	0.33	0.33
<i>M. pachydermatis</i>	011000100		0.33	0.33	0.77	0.55	0.55
<i>M. sympodialis</i>	100001001			0.55	0.33	0.33	0.33
<i>M. slooffiae</i>	100100010				0.55	0.55	0.55
<i>M. obtusa</i>	011000010					0.77	0.77
<i>M. globosa</i>	010010010						1.00 ^c
<i>M. restricta</i>	010010010						

^a PCR-REA types that resulted from the analysis of the LSU of the rRNA gene and the ITS have been used to construct these multilocus genotypes. Only the common representative PCR-REA types for each *Malassezia* species are included. The sequence of PCR-REA types is ABCC'DEFGH; 1 represents the presence and 0 represents the absence of a particular PCR-REA type.

^b Similarity indices are calculated on the basis of the number of matches between all pairs of *Malassezia* species. Either the presence or absence of a particular PCR-REA type is considered a match.

^c *M. globosa* and *M. restricta* could be distinguished only by TD-PCR, using primers for the β -tubulin gene.

yielding an ~550-bp fragment, whereas those of *M. globosa* were never amplified (Fig. 3). Other species gave inconsistent results (Tables 1 and 3).

Lipase gene. The lipase gene region was of primary interest to us because of basic differences in the lipid requirements among *Malassezia* species. While six of the seven *Malassezia* species require exogenous lipids for growth, *M. pachydermatis* can grow in media without additional lipids. Since the lipase gene of *Malassezia* has not been characterized molecularly, we designed primers from the published sequence of the *LIP2* gene of *Candida cylindracea* (24). Lipase sequences of *C. cylindracea* were used because the lipase activities and pH profiles of *M. furfur* and *C. cylindracea* lipases have been shown to be similar (30). On annealing at 50°C, these primers resulted in several nonspecific bands for different *Malassezia* species that were not reproducible, whereas fragments of ~850 and <1,200 bp were consistently amplified for the two *M. pachydermatis* strains included in this study. Annealing at 55°C resulted in a product of ~560 bp (close to the expected size [Table 2]) from two *M. pachydermatis* strains, CBS 1879 and ATCC 14521, as well as from strain GM420. At 55°C annealing, the one *M. globosa* strain tested (98F-8304) revealed a product of <1,200 bp, whereas for the remaining five *Malassezia* species, no amplification product was observed (data not shown). These preliminary results suggest that this region might be useful in identification of *M. pachydermatis* and *M. globosa* strains.

DISCUSSION

The data presented in this study conform to the present nomenclature of *Malassezia* yeasts. Our results show that PCR-REA may provide a rapid and reliable technique for molecular differentiation of *Malassezia* species. However, the status of a small number of isolates remained ambiguous after PCR-REA typing, and these techniques should be employed as a useful adjunct to conventional testing until additional study resolves the correct placement of the anomalous isolates.

M. furfur, *M. sympodialis*, and *M. slooffiae* are physiologically very similar, and ambiguity remained regarding correct identification of these species on the basis of tests for utilization of tween compounds in the simple media (17). Recently, Mayer et al. (28) have reported the use of additional tests, such as addition of cremophor EL in diffusion plates and characterization of β -glucosidase activity, to resolve this ambiguity. In the present study, PCR-RFLP analysis of only one genomic region, the ITS region, proved sufficient to resolve the ambiguity between the three physiologically similar species. Similarly,

M. obtusa, which is similar in growth requirements to *M. globosa* and *M. restricta*, could be differentiated from these species by variation in the ITS region. *M. globosa* and *M. restricta*, however, which are readily distinguishable by differences in cell morphology and catalase activity (14), were difficult to distinguish on the basis of variation in ribosomal DNA.

Although we believe that the PCR-REA system is generally very reliable, we recommend that a microscopic examination of yeast cells and a catalase test should also be done for an accurate identification. The four discrepancies observed among species identifications on the basis of physiological and molecular tests suggest that for some ambiguous cases it may become necessary to conduct additional tests, such as a test for growth on Sabouraud agar to distinguish *M. pachydermatis* from *M. furfur*. The presence of more than one PCR-REA type, in most cases, can be explained by either gain or loss of a restriction site, with the only exception being type A' of the LSU region, which is indicative of a recombinant strain. The presence of more than one PCR-REA type for *M. furfur*, *M. sympodialis*, and *M. slooffiae* is suggestive of intraspecific variation, but such

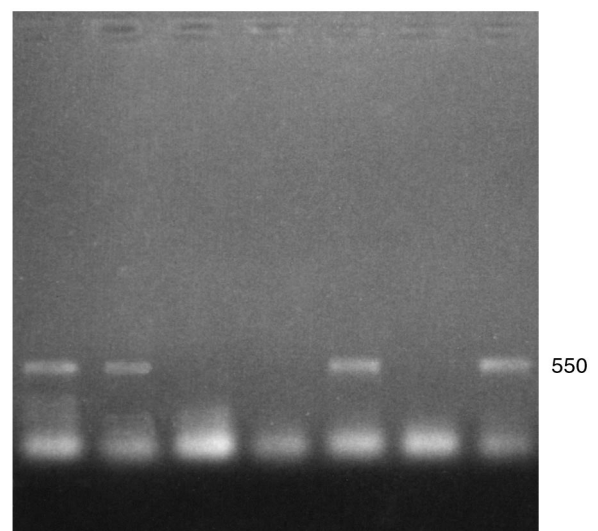


FIG. 3. Hot start-TD PCR amplification product obtained using primers for the β -tubulin gene (12). Lanes (left to right): YKM 31 (*M. restricta*), CBS 7877 (*M. restricta*), 98F 9925 (*M. sympodialis*), 98F-7317 (*M. globosa*), JF4 (*M. furfur*); 98F-8316 (*M. obtusa*), and CBS 7956 (*M. slooffiae*). Positive amplification of a band of ~550 bp is seen in lanes 1, 2, 5, and 7.

intraspecific variation, if present, has to be confirmed further with additional screening.

The results of this study are comparable with those of Guillot and Guého (16), who, in an rRNA sequence study of *Malassezia* species, observed unique sequences for *M. sympodialis*. In the present study as well, *M. sympodialis* was readily distinguishable from other species on the basis of a smaller amplicon (~700 bp) for the ITS region. Guillot and Guého (16) found that when the most variable D2 region of the ribosomal gene was examined, maximal divergence was observed between sequences for *M. furfur* and *M. restricta*. We also found that *M. furfur* was most divergent from *M. restricta* and *M. globosa*, with only 33% similarity among these species on the basis of the molecular markers screened.

The neotype strain of *Pityrosporium ovale* (CBS 1878), currently identified as *M. furfur*, is atypical culturally and serologically (9) as well as karyotypically (6). Its anomalous nature is confirmed in this study. PCR amplification of CBS 1878 with primers for LSU region followed by restriction analysis revealed in a PCR-REA type that was different from that of the other representative strains of *M. furfur*.

Karyotyping, although very reliable for molecular differentiation of *Malassezia* species, takes at least 60 to 72 h for analysis of each sample after culture growth and isolation of protoplasts. In contrast, the PCR-RFLP analysis reported here can be completed in less than 15 h after DNA extraction. Once the extraction protocol is optimized for direct DNA extractions from skin scrapings, the 2 to 5 days required for appreciable yeast growth in culture can be eliminated. It may thus be possible, after further development, to provide reliable species identifications back to the physician in less than 3 days. Thus, the PCR-RFLP procedure may ultimately prove to be preferable to both karyotyping and culture analysis for identification of *Malassezia* species.

PCR-RFLP analysis for other regions or protein-encoding genes may provide further insight into intraspecific variation and thus be very useful in epidemiological studies.

REFERENCES

- Anaissie, E., and G. P. Bodey. 1989. Nosocomial fungal infections: old problems and new challenges. *Infect. Dis. Clin. N. Am.* **3**:867-882.
- Anthony, R. M., S. A. Howell, and L. Pintors. 1994. Application of DNA typing methods to the study of the epidemiology of *Malassezia pachydermatis*. *Microb. Ecol. Health Dis.* **7**:161-168.
- Ault, G. S., C. F. Ryschkewitsch, and G. L. Stoner. 1994. Type-specific amplification of viral DNA using touchdown and hotstart PCR. *J. Virol. Methods* **46**:145-156.
- Belkum, A., T. Boekhout, and R. Bosboom. 1994. Monitoring spread of *Malassezia* infections in a neonatal intensive care unit by PCR-mediated genetic typing. *J. Clin. Microbiol.* **32**:2528-2532.
- Boekhout, T., and R. W. Bosboom. 1994. Karyotyping of *Malassezia* yeasts: taxonomic and epidemiological implications. *Syst. Appl. Microbiol.* **17**:147-153.
- Boekhout, T., M. Kamp, and E. Guého. 1998. Molecular typing of *Malassezia* species with PFGE and RAPD. *Med. Mycol.* **36**:365-372.
- Carbone, I., and L. M. Kohn. 1993. Ribosomal DNA sequence divergence within internal transcribed spacer 1 of the Sclerotiniaceae. *Mycologia* **85**:415-427.
- Carlotti, A., P. Chaib, A. Couble, N. Bourgeois, V. Blanchard, and J. Villard. 1997. Rapid identification and fingerprinting of *Candida krusei* by PCR-based amplification of the species-specific repeated polymorphic sequence CKRS-1. *J. Clin. Microbiol.* **35**:1337-1343.
- Cunningham, A. C., J. P. Leeming, E. Ingham, and G. Gowland. 1990. Differentiation of three serovars of *Malassezia furfur*. *J. Appl. Bacteriol.* **68**:439-446.
- Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick. 1991. Touchdown PCR to circumvent spurious priming gene amplification. *Nucleic Acids Res.* **19**:4008.
- Geogiev, O. I., N. Nikolaev, A. A. Hadjiolov, K. G. Skryabin, N. M. Zakharyev, and A. A. Bayev. 1981. The structure of the yeast ribosomal genes. 4. Complete sequence of the 25S rRNA gene from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **9**:6953-6958.
- Glass, N. L., and G. C. Donaldson. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* **61**:1323-1330.
- Guého, E., and S. A. Meyer. 1989. A reevaluation of the genus *Malassezia* by means of genome comparison. *Antonie Leeuwenhoek* **55**:245-251.
- Guého, E., G. Midgley, and J. Guillot. 1996. The genus *Malassezia* with description of four new species. *Antonie Leeuwenhoek* **69**:337-355.
- Guého, E., T. Boekhout, H. R. Ashbee, J. Guillot, A. Van Belkum, and J. Faergemann. 1998. The role of *Malassezia* species in the ecology of human skin and as pathogens. *Med. Mycol.* **36**:220-229.
- Guillot, J., and E. Guého. 1995. The diversity of *Malassezia* yeasts confirmed by rRNA sequence and nuclear DNA comparisons. *Antonie Leeuwenhoek* **67**:297-314.
- Guillot, J., E. Guého, M. Lesourd, G. Midgley, G. Chévrier, and B. Dupont. 1996. Identification of *Malassezia* species: a practical approach. *J. Mycol. Med.* **6**:103-110.
- Hazen, K. C. 1995. New and emerging yeast pathogens. *Clin. Microbiol. Rev.* **8**:462-478.
- Jackson, C., R. C. Barton, and G. V. Evans. 1999. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. *J. Clin. Microbiol.* **37**:931-936.
- Kiuchi, A., S. Taharaguchi, R. Hanazawa, M. Hara, T. Ikeda, and K. Tabuchi. 1992. Chromosome-sized DNA of *Malassezia pachydermatis* by pulsed-field gel electrophoresis. *J. Vet. Med. Sci.* **54**:1219-1220.
- Leclerc, M. C., H. Philippe, and E. Guého. 1994. Phylogeny of dermatophytes and dimorphic fungi based on large subunit ribosomal RNA sequence comparisons. *J. Med. Vet. Mycol.* **32**:331-341.
- Lee, C.-H., J. Helweg-Larsen, X. Tang, S. Jin, B. Li, M. S. Barlett, and J.-J. Liu. 1998. Update on *Pneumocystis carinii* f. sp. *hominis* typing based on nucleotide sequence variations in internal transcribed spacer regions of rRNA genes. *J. Clin. Microbiol.* **36**:734-741.
- Leeming, J. P., and F. H. Notman. 1987. Improved methods for isolation and enumeration of *Malassezia furfur* from human skin. *J. Clin. Microbiol.* **25**:2017-2019.
- Longhi, S., F. Fuseth, R. Gordon, M. Lotti, M. Vanoni and L. Alberghina. 1992. Cloning and nucleotide sequences of two Lip genes from *Candida cylindracea*. *Biochim. Biophys. Acta* **1131**:227-232.
- Makimura, K., S. Y. Murayama, and H. Yamaguchi. 1994. Detection of a wide range of medically important fungi by polymerase chain reaction. *J. Med. Microbiol.* **40**:358-364.
- Makimura, K., Y. Tamura, T. Mochizuki, A. Hasegawa, Y. Tajiri, R. Hanazawa, K. Uchida, H. Saito, and H. Yamaguchi. 1999. Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. *J. Clin. Microbiol.* **37**:920-924.
- Marcon, M. J., and D. A. Powell. 1992. Human infections due to *Malassezia* spp. *Clin. Microbiol. Rev.* **5**:101-119.
- Mayer, P., P. Haze, C. Papavassilis, M. Pickel, K. Gruender, and E. Guého. 1997. Differentiation of *Malassezia* species: selectivity of cremophor EL, castor oil and ricinoleic acid for *M. furfur*. *Br. J. Dermatol.* **137**:208-213.
- Radford, S. A., E. M. Johnson, J. P. Leeming, M. R. Millar, J. M. Cornish, A. B. M. Foot, and D. W. Warnock. 1998. Molecular epidemiological study of *Aspergillus fumigatus* in a bone marrow transplantation unit by PCR amplification of ribosomal intergenic spacer sequences. *J. Clin. Microbiol.* **36**:1294-1299.
- Ran, Y., T. Yoshike, and H. Ogawa. 1993. Lipase of *Malassezia furfur*: some properties and their relationship to cell growth. *J. Med. Vet. Mycol.* **31**:77-85.
- Rinaldi, M. G. 1989. Emerging opportunists. *Infect. Dis. Clin. N. Am.* **3**:65-76.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Samonis, G., and D. Bafaloukos. 1992. Fungal infections in cancer patients: an escalating problem. *In Vivo* **6**:183-194.
- Sansinforiano, M. E., J. A. Padilla, H. de Mendoza, M. H. de Mendoza, J. L. Fernandez-Garcia, M. Martinez-Trancon, A. Rabasco, and J. C. Parejo. 1998. Rapid and easy method to extract and preserve DNA from *Cryptococcus neoformans* and other pathogenic yeasts. *Mycoses* **41**:195-198.
- White, T. J., T. Burns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), *PCR protocols: a guide to methods and applications*. Academic Press, San Diego, Calif.