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Identification and Typing of *Malassezia* Species by Amplified Fragment Length Polymorphism and Sequence Analyses of the Internal Transcribed Spacer and Large-Subunit Regions of Ribosomal DNA

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***Malassezia* yeasts are associated with several dermatological disorders. The conventional identification of *Malassezia* species by phenotypic methods is complicated and time-consuming, and the results based on culture methods are difficult to interpret. A comparative molecular approach based on the use of three molecular techniques, namely, amplified fragment length polymorphism (AFLP) analysis, sequencing of the internal transcribed spacer, and sequencing of the D1 and D2 domains of the large-subunit ribosomal DNA region, was applied for the identification of *Malassezia* species. All species could be correctly identified by means of these methods. The results of AFLP analysis and sequencing were in complete agreement with each other. However, some discrepancies were noted when the molecular methods were compared with the phenotypic method of identification. Specific genotypes were distinguished within a collection of *Malassezia furfur* isolates from Canadian sources. AFLP analysis revealed significant geographical differences between the North American and European *M. furfur* strains.**

The genus *Malassezia* has received considerable attention in recent years from dermatologists and other clinicians. This group of basidiomycetous yeasts, long known to be the causal agents of pityriasis (tinea) versicolor, is also increasingly being associated with the causation of folliculitis, papillomatosis, and invasive human infections, as well as potential immunogenic triggering of atopic dermatitis, seborrheic dermatitis, and dandruff (6, 9, 11, 14, 18, 25, 26, 29). *Malassezia* species are listed among the new and emerging yeast pathogens (22, 24).

The genus *Malassezia*, until recently, was characterized on the basis of rRNA sequences as consisting of seven species, including the lipid-dependent species *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta*, and *M. slooffiae* and the lipophilic species *M. pachydermatis* (12, 13). Recently, two new species have been identified: *M. dermatis* (39), which was isolated from atopic dermatitis patients, and *M. equi* (unpublished), a species that was isolated from the skin of horses (32). The latter species has only tentatively been named and still awaits formal description; the present study deals only with the eight described species.

Guillot et al. (17) introduced a physiological system based on lipid assimilation and other phenotypic characteristics for identifying the various *Malassezia* species. This phenotypic system has been used as the conventional method of identification, though in practice, the test results are not always easy to read. Therefore, several research groups have explored the use of molecular techniques, such as pulsed-field gel electrophoresis (3, 4, 36), randomly amplified polymorphic DNA analysis (1, 3), amplified fragment length polymorphism (AFLP) analysis (40), denaturing gradient gel electrophoresis (40), multilocus enzyme electrophoresis (30), sequencing analysis (37), restriction analysis of PCR amplicons of ribosomal sequences (8, 15, 16, 20, 28), and chitin synthase gene sequence analysis (1, 4, 8, 37), for the identification of *Malassezia* species. Recently, Gemmer et al. (9) devised a remarkably efficient, novel technique, terminal fragment length polymorphism analysis, for the rapid and reliable identification of *Malassezia* species. It eliminates the need for strain cultivation in direct investigations of *Malassezia* populations on skin samples, as well as the need for restriction enzyme digestion of nucleic acids. The technique, however, is not suitable for epidemiological typing, as its ability to show heterogeneity within a species is limited.

The objective of the present study was to facilitate an improved understanding of the epidemiology of *Malassezia* infections by determining which molecular characterization methodologies could most effectively be used for the identification and strain typing of *Malassezia* species. It is important to identify the different species correctly, as some studies have given preliminary indications that different *Malassezia* species may occupy well-defined niches on the human body and may play differential roles in causing various diseases (11, 19, 21). However, these studies have not all been in agreement about which species is prevalent or etiologically important with regard to a specific disease, suggesting that technical improvements in identification methods is critical in producing definitive results.

AFLP analysis is a universally applicable technique that has proven to be useful for the identification and strain typing of microorganisms (2, 5, 34). In this study, we investigated the use of AFLP analysis as well as rapid sequencing of the internal transcribed spacer (ITS) and large-subunit (LSU) regions of the nuclear ribosomal DNA (rDNA) for the identification of the eight currently recognized *Malassezia* species. We compared the two molecular methods with the conventional meth-

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od of identification to see if any discrepancies could be observed. The molecular techniques were used to investigate the genetic diversity of *Malassezia* isolates obtained from a variety of patients in Canada. These patients were mostly from the province of Ontario, a region with a notably diverse population developed through immigration from many areas of the world. In order to understand the extent to which the Ontario isolates might reflect the overall genetic biodiversity of *Malassezia* spp. from human sources and to ensure that the techniques used were maximally developed as tools for identifying genetic types within *Malassezia* species, a diverse comparison sample of *Malassezia* strains from different worldwide geographic regions was chosen from the collection of the Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands).

MATERIALS AND METHODS

Yeast strains. One hundred four isolates representing the eight currently recognized *Malassezia* species were studied. The origins, locations, and hosts of the strains are listed in Table 1. Sixty-five of the strains had originally been isolated and phenotypically identified (17) at the Mycology Laboratory, Ontario Ministry of Health, Toronto, Ontario, Canada, and at Mediprobe Laboratories Inc., London, Ontario, Canada. Thirty-nine isolates were taken from the collection of the CBS. Three strains were received as a gift from Gillian Midgley (London, United Kingdom), and one strain was received from Jan Faergemann (Göteborg, Sweden). Six of the study strains had been isolated from patients residing in Hawaii, South Africa, and Hong Kong. The strains were maintained at 30°C on Leeming and Notman medium, consisting of 1% peptone, 0.5% glucose, 0.01% yeast extract, 0.4% desiccated ox bile, 0.1% (vol/vol) glycerol, 0.5% glycerol monostearate, 0.05% (vol/vol) Tween 60, 1% (vol/vol) high-fat cow's milk, and 1.5% agar in distilled water. The percentages given are for weight per volume unless otherwise specified.

DNA extraction. DNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method (33) from 4- to 5-day-old cultures. Briefly, 3 loopfuls of yeast growth was transferred to a 1.5-ml microcentrifuge tube containing about 100 mg of sterile sand and 800 μl of CTAB buffer (33). Cells were disrupted mechanically for approximately 1 min by using a pestle. The mixture was vortexed and incubated for 4 h at 65°C. The suspension was centrifuged for 30 min at 18,300 × *g* rpm at 4°C, and 700 μl of the supernatant was transferred to a fresh microcentrifuge tube. Subsequently, 700 μl of chloroform-isoamyl alcohol (24:1 by volume) was added, and the solution was shaken vigorously. The solution was centrifuged at 18,300 × *g* for 20 min at 4°C, and 500 μl of the supernatant was transferred to a fresh tube. To this, 500 μl of chloroform-isoamyl alcohol was added, and the suspension was centrifuged again at 200 × *g* for 10 min at 4°C. From this suspension, 350 μl of the aqueous layer was taken and mixed with 150 μl of CTAB buffer. To this, 300 μl of ice-cold isopropanol (kept at −20°C) was added, and the DNA was precipitated by centrifuging at 18,300 × *g* for 10 min at 4°C. The pellet obtained was washed with cold 70% ethanol. After being dried, the pellet was suspended in 100 μl of sterile water plus 4 μl of RNase (10 mg/ml) (USB Corp., Cleveland, Ohio). The samples were stored at −20°C.

AFLP analysis. AFLP analysis was performed according to the manufacturer's instructions in the AFLP microbial fingerprinting protocol (PE Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), with some modifications. Restriction and ligation were performed simultaneously on 10 ng of genomic DNA by using 1 U of MseI, 5 U of EcoRI, and 3 U of T4 DNA ligase (Biolabs, Westburg, The Netherlands). The sequences of the primers EcoRI and MseI were 5′-GA CTGCGTACCAATTCAC-3′ and 5′-GATGAGTCCTGAGTAAC-3′, respectively. The adaptors used were EcoRI (5′-CTCGTAGACTGCGTACC-3′, forward; 3′-CATCTGACGCATGGTTAA-5′, reverse) and MseI (5′-GACGATG AGTCCTGAG-3′, forward; 3′-CTACTCAGGACTCAT-5′, reverse). The reaction was allowed to take place in a total volume of 5.5 μl with the following constituents: a 0.36 μM concentration of the EcoRI adaptor and a 3.64 μM concentration of the MseI adaptor 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 of bovine serum albumin ml^{-1}. The restriction ligation mixture was incubated for 2 h at 37°C and later diluted by adding 25 μl of sterile double-distilled water. The first PCR was performed with two preselective primers (EcoRI core sequence and MseI core sequence) and the AFLP amplification core mix from the AFLP microbial fingerprinting kit, according to the manufacturer's 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 of sterile double-distilled water. The second PCR used more-selective primers, EcoRI-A FAM and MseI-G. The conditions were 2 min at 94°C; 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; and then 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 capillary electrophoresis with the following loading mix: 2.0 μl of selective amplification product, 24 μl of deionized formamide, and 1 μl of GeneScan-500 labeled with 6-carboxy-X-rhodamine (Applied Biosystems, Foster City, Calif.) as an internal size standard. After incubation for 5 min at 95°C, the samples were run on an ABI 310 genetic analyzer for 30 min each. Data were analyzed with the Bionumerics software package (version 2.5; Applied Maths, Kortrijk, Belgium), by using (i) Pearson correlation based on similarities of the densitometric curves and (ii) the unweighted pair group method with arithmetic means analysis. The statistical reliability of the clusters was confirmed by using the cophenetic values, which calculate the correlation between the calculated similarities and the dendrogram-derived similarities.

PCR amplification and sequencing of ITS and LSU regions. The ITS I and ITS II regions and the D1 and D2 domains of the LSU region were amplified with primers V9 (5′-TGCGTTGATTACGTCCCTGC) and RLR3R (5′-GGTCCGT GTTTCAAGAC). The PCR conditions were 5 min at 94°C; 35 cycles of 45 s at 94°C, 40 s at 56°C, and 2 min at 72°C; followed by chilling at 4°C. The PCR products were purified by using GFX columns (Amersham Pharmacia Biotech Inc., Roosendaal, The Netherlands) and visualized on electrophoresis gel after ethidium bromide staining. The rDNA was sequenced with the BigDye terminator cycle sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 3700 sequencer (Applied Biosystems) by using the standard conditions recommended by the vendor. The primers used in the sequence reaction were ITS4 (5′-TCCTCCGCTTATTGATATGC) and ITS5 (5′-GGAAGTAAAAGTCGTA ACAAGG) for analysis of the ITS region and NL1 (5′-GCATATCAATAAGC GGAGGAAAAG) and RLR3R (5′-GGTCCGTGTTTCAAGAC) for analysis of the D1 and D2 domains of 26S rDNA. The PCR conditions were as follows: 25 PCR cycles of 96°C for 10 s (denaturation), 50°C for 5 s (annealing), and 60°C for 4 min (extension). The sequencing products were purified with Sephadex (Amersham Pharmacia). Sequences were assembled and edited with Seqman II software (DNAStar Inc., Madison, Wis.) and aligned with Megalign (DNAStar). The sequences were visually corrected. Phylogenetic analysis was performed by using PAUP (version 4) parsimony analysis, random stepwise addition, and tree bisection-reconnection.

Statistical analysis. Likelihood ratios were computed by using SPSS for Macintosh (release 11.0.2, 2003; SPSS, Chicago, Ill.).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in GenBank. The GenBank numbers for D1 and D2 domain and ITS sequences are listed in Table 1.

RESULTS

AFLP analysis. (i) Identification of species. The dendrogram obtained by unweighted pair group method with arithmetic means analysis is shown in Fig. 1. The eight *Malassezia* species could clearly be distinguished in the AFLP-based tree (Fig. 1). The tree comprised nine main groups, or clusters, and within those clusters, those subtending *M. furfur*, *M. slooffiae*, and *M. globosa* had distinct phylogenetic substructures. Genotypes were assigned to each cluster as shown in Fig. 1. The cophenetic and similarity values for various groups are shown in Table 2.

Some interesting observations were made for the *M. furfur* cluster. AFLP patterns revealed that considerable genetic diversity exists within this species. Eight well-distinguished subtypes were revealed. Strains from subtype 3 of this species as delineated by Theelen et al. (40) were not included in the present study, but this subtype designation has been retained to avoid confusion. The distribution of the 31 *M. furfur* isolates studied across the subtypes discussed here was relatively even, a circumstance which, unfortunately, limited the sample number per subtype, thus making analysis problematic. Nonethe-

TABLE 1. *Malassezia* isolates included in the study

Isolate[a]	Origin	Location	AFLP subtype[b]	Host	ITS accession no.	LSU accession no.	% Similarity of ITS sequences	% Similarity of LSU sequences
M. furfur								
CBS 1878, NT of *Pityrosporum ovale**	Dandruff	Unknown	2	Human	AY387100	AY387196	100	100
EL8 = CBS 9365	Skin	France	2	Elephant	AY387101	AY387197	100	100
CBS 4172	Skin	Sweden	2	Elk	AY387102	AY387198	100	100
CBS 7969	Skin	France	2	Elephant	AY387103	AY387199	100	100
WF8 = CBS 9371	Arm of tinea versicolor patient	Hawaii	2	Human	AY387104	AY387200	100	100
98F8480 = CBS 9376	Lesion on skin	Canada	1	Human	AY387123	AY387219	100	100
2ATB1	Back	Canada	1	Human	AY387124	AY387220	98.62	99.64
SWB1 = CBS 9371	Back	Canada	1	Human	AY387125	AY387221	98.62	99.64
TPB1 = CBS 9372	Back	Canada	1	Human	AY387126	AY387222	98.62	99.64
SOS3 = CBS 9369	Scalp	Canada	1	Human	AY387127	AY387223	98.62	99.64
CBS 7982	Skin of ear from healthy subject	France	1	Human	AY387128	AY387224	98.62	99.64
4SJCMC1 = CBS 9374	Chest	Canada	1	Human	AY387131	AY387227	98.62	99.64
PM312	Urine of neonate	Germany	4	Human	AY387118	AY387214	100	100
PM314	Tracheal secretion	Germany	4	Human	AY387119	AY387215	100	100
PM316	Throat of neonate	Germany	4	Human	AY387120	AY387216	100	100
JLPK23	Catheter, blood	France	4	Human	AY387121	AY387217	99.59	100
JLPK18	Catheter, liver recipient, blood	France	4	Human	AY387122	AY387218	99.59	100
GM420 = CBS 9367	Unknown	United Kingdom	5	Human	AY387105	AY387201	100	100
JLPK13	Urine	France	5	Human	AY387106	AY387202	99.59	100
CBS 7860	Skin of ear from neonate	United Kingdom	5	Human	AY387114	AY387210	99.59	100
CBS 7867	Skin of ear from neonate	United Kingdom	5	Human	AY387115	AY387211	99.59	100
CBS 7710	Skin	The Netherlands	5	Human	AY387117	AY387213	100	99.64
HK8 = CBS 9366	Skin	Hong Kong	6	Human	AY387107	AY387203	98.9	100
NCF3 = CBS 9364	Forehead	Canada	6	Human	AY387108	AY387204	98.9	100
NCB3 = CBS 9368	Back	Canada	6	Human	AY387109	AY387205	98.9	100
NCC2 = CBS 9375	Chest	Canada	6	Human	AY387110	AY387206	98.9	100
MF9	Forehead	Canada	6	Human	AY387111	AY387207	100	100
NCF2 = CBS 9373	Forehead	Canada	6	Human	AY387112	AY387208	98.9	99.82
CBS 6000	Dandruff	India	7	Human	AY387113	AY387209	100	99.64
CBS 7985	Wing of ostrich	France	8	Ostrich	AY387129	AY387225	98.62	99.82
CBS 7984	Ear of healthy elephant	France	8	Elephant	AY387130	AY387226	98.48	99.82
M. globosa								
CBS 7966 T*	Pityriasis versicolor	United Kingdom		Human	AY387132	AY387228	100	100
CBS 7874	Dandruff	United Kingdom		Human	AY387133	AY387229	92.9	99.82
HK10	Skin	Hong Kong		Human	AY387134	AY387230	95.49	100
MPS3	Scalp	Canada		Human	AY387135	AY387231	94.98	99.82
CBC1	Chest	Canada		Human	AY387136	AY387232	95.37	99.82
M. obtusa								
CBS 7876 T, GM 215*	Skin	United Kingdom		Human	AY387137	AY387233	100	100
CBS 7968	Atopic dermatitis	United Kingdom		Human	AY387138	AY387234	100	100
M. pachydermatis								
CBS 1879, NT of *M. pachydermatis**	Skin	France		Pig	AY387139	AY387235	100	100
CBS 1919	Ulcerated ear of dog	Europe		Dog	AY387140	AY387236	100	100
CBS 1885	Ear of dog with otitis extema	Sweden		Dog	AY387141	AY387237	95.27	99.46
CBS 1884	Ear of dog with otitis extema	Sweden		Dog	AY387142	AY387238	100	100
M. restricta								
CBS 7877, T*	Healthy skin	United Kingdom		Human	AY387143	AY387239	100	100
CBS 8747	Healthy scalp	Canada		Human	AY387144	AY387240	99.85	100
CBS 7991	Normal skin	United Kingdom		Human	AY387145	AY387241	90.32	99.46
M. slooffiae								
CBS 7956 T, JG 554*	Healthy ear	France		Pig	AY387146	AY387242	100	100
CBS 7973	Pityriasis versicolor	France		Human	AY387147	AY387243	99.25	100
RBF2	Forehead	Canada		Human	AY387148	AY387244	99.11	100
CBS 7975	Dandruff	United Kingdom		Human	AY387149	AY387245	98.96	100
CBS 7875	Dandruff	United Kingdom		Human	AY387150	AY387246	99.23	87.84
CBS 7972	Pityriasis versicolor	France		Human	AY387151	AY387247	99.25	100
CBS 7971	Scalp	United Kingdom		Human	AY387152	AY387248	99.7	99.82
TV1	Back	Canada		Human	AY387153	AY387249	98.94	100
AWC3	Chest	Canada		Human	AY387154	AY387250	99.7	99.82
GM150	Unknown	United Kingdom		Human	AY387155	AY387251	99.49	99.82
SF2	Skin	South Africa		Human	AY387156	AY387252	99.1	99.82

Continued on following page

TABLE 1—*Continued*

Isolate[a]	Origin	Location	AFLP subtype[b]	Host	ITS accession no.	LSU accession no.	% Similarity of ITS sequences	% Similarity of LSU sequences
M. sympodialis								
CBS 7222 T, EG 604*	Human ear	United States		Human	AY387157	AY387253	100	100
WBC2	Chest	Canada		Human	AY387158	AY387254	100	100
2SMC3	Chest	Canada		Human	AY387159	AY387255	99.52	100
ZHC1	Chest	Canada		Human	AY387160	AY387256	100	100
VMB3	Back	Canada		Human	AY387161	AY387257	100	100
98F9925	Lesion on trunk	Canada		Human	AY387162	AY387258	99.52	100
WF39	Patient with folliculitis	Hawaii		Human	AY387163	AY387259	99.52	100
LNC1	Chest	Canada		Human	AY387164	AY387260	99.52	100
ZHC3	Chest	Canada		Human	AY387165	AY387261	99.52	100
3SKB3	Back	Canada		Human	AY387166	AY387262	99.84	100
RBB1	Back	Canada		Human	AY387167	AY387263	99.52	100
2MBB1	Back	Canada		Human	AY387168	AY387264	100	100
SSB2	Back	Canada		Human	AY387169	AY387265	99.52	100
2CC3	Chest	Canada		Human	AY387170	AY387266	100	100
98F8139	Lesion on skin	Canada		Human	AY387171	AY387267	100	100
LMB3	Back	Canada		Human	AY387172	AY387268	99.52	100
RPC1	Chest	Canada		Human	AY387173	AY387269	99.52	100
RBC1	Chest	Canada		Human	AY387174	AY387270	100	100
KEB1	Back	Canada		Human	AY387175	AY387271	99.52	100
LGB3	Back	Canada		Human	AY387176	AY387272	99.52	100
RGC2	Chest	Canada		Human	AY387177	AY387273	100	100
DKC1	Chest	Canada		Human	AY387178	AY387274	100	100
RPB2	Back	Canada		Human	AY387179	AY387275	99.52	100
JF05	Back of healthy 23-year-old female	Sweden		Human	AY387180	AY387276	100	100
WF42	Back of patient with folliculitis	Hawaii		Human	AY387181	AY387277	100	100
WBB2	Back	Canada		Human	AY387182	AY387278	100	100
3SKB1	Back	Canada		Human	AY387183	AY387279	99.52	100
AEB2	Back	Canada		Human	AY387184	AY387280	99.84	100
BMB3	Back	Canada		Human	AY387185	AY387281	100	100
CBS 7979	Skin	United Kingdom		Human	AY387186	AY387282	99.52	100
CBS 7978	Pityriasis versicolor	United Kingdom		Human	AY387187	AY387283	99.52	100
ZHB2	Back	Canada		Human	AY387188	AY387284	100	100
CWB1	Back	Canada		Human	AY387189	AY387285	99.52	100
GM323	Unknown	United Kingdom		Human	AY387190	AY387286	100	100
RBC3	Chest	Canada		Human	AY387190	AY387287	100	100
CWB2	Back	Canada		Human	AY387192	AY387288	99.52	100
GMB1	Back	Canada		Human	AY387193	AY387289	100	100
JSB2	Back	Canada		Human	AY387194	AY387290	99.52	100
98F	Lesion on skin	Canada		Human	AY387195	AY387291	100	100
CBS 7865	Skin	United Kingdom		Human	AY387116	AY387212	99.59	100
M. dermatis								
CBS 9145	Atopic dermatitis	Japan		Human	AB070360	AB070365	100	100
CBS 9169	Atopic dermatitis	Japan		Human	AB070356	AB070361	100	100
CBS 9170	Atopic dermatitis	Japan		Human	AB070358	AB070363	100	100
Unknown species								
ISB2	Back	Canada		Human	Unknown	Unknown	Unknown	Unknown
LNS2	Scalp	Canada		Human	Unknown	Unknown	Unknown	Unknown
KHS2	Scalp	Canada		Human	Unknown	Unknown	Unknown	Unknown
BSB1	Back	Canada		Human	Unknown	Unknown	Unknown	Unknown
TPF2	Forehead	Canada		Human	Unknown	Unknown	Unknown	Unknown
DRC2	Chest	Canada		Human	Unknown	Unknown	Unknown	Unknown
4GMC1	Chest	Canada		Human	Unknown	Unknown	Unknown	Unknown

[a] Asterisks indicate the type strains used for determining similarity values.
[b] AFLP subtypes are shown for *M. furfur* only.

less, it can be noted that just two subtypes, types 2 and 8, contained all five zoonotic isolates tested, including strains from elephant, elk, and ostrich. Subtype 2 also contained two isolates from human skin. Subtypes 1 and 6 contained the majority (13 of 17) of the skin (including scalp) isolates obtained from nonneonatal humans. Two skin isolates from the ears of neonates, however, were found to belong to subtype 5, which also contained one isolate from nonneonatal skin. Subtype 4 appeared remarkable in that all five isolates that were available were from internal body sites, catheter sites, or mucosae rather than from healthy skin; two of these isolates were recorded as coming from neonates. Subtype 5, apart from containing the two neonatal ear skin isolates previously mentioned, contained the only other internal body site isolate examined, an isolate from urine. Of the isolates obtained in connection with hospitals (including neonatal wards) and/or systemic disease, then, all belonged to subtypes 4 and 5, and at least 8 of 10 isolates in these subtypes were derived from such

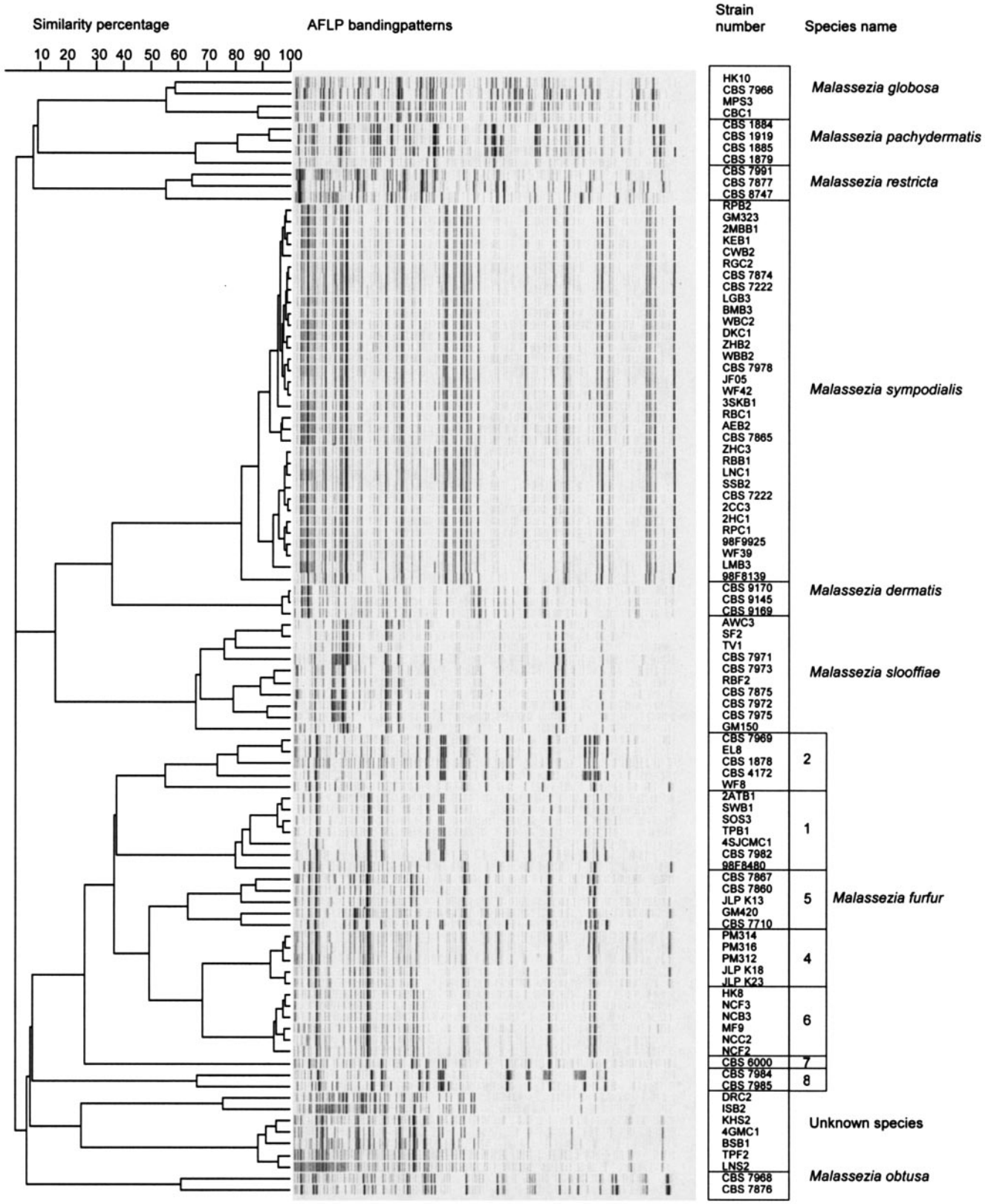

FIG. 1. AFLP analysis banding patterns of *Malassezia* species. Unknown species, strains not associated with any recognized *Malassezia* taxon.

TABLE 2. Cophenetic and similarity values for various clusters of *Malassezia* spp. based on comparisons with type strain of each species

Species and cluster no.	No. of strains	% Similarity value	Cophenetic value
M. furfur	29	26.49	85
Cluster 1	4	74.15	94
Cluster 2	1	55.27	90
Cluster 3	7	80.52	83
Cluster 4	5	63.37	93
Cluster 5	5	93.07	95
Cluster 6	6	94.37	84
Cluster 7	1	26.49	85
M. sympodialis	33	82.39	79
Cluster 1	21	74.88	78
Cluster 2	11	82.39	79
Cluster 3	1	82.39	79
M. dermatis	3	97.33	90
M. globosa	4	55.15	98
M. pachydermatis	4	65.81	94
M. restricta	3	55.41	92
M. slooffiae	10	66.33	74
Cluster 1	4	76.37	93
Cluster 2	5	79.65	84
Cluster 3	1	66.33	74
M. obtusa	2	61.19	100
Unknown species			
Cluster 1	2	75.95	100
Cluster 2	5	88.83	66

sources. The source of subtype 5 isolate GM 420/CBS 9367 has not been traced.

Because many of the subtype 1 and 6 isolates were from the trunk (back and chest), we tested whether these genotypes might be significantly associated with this body site. Isolate numbers permitted statistical testing for the significance of differences in *M. furfur* subtypes among isolates from the trunk, the head (including the scalp and ear, whether neonatal or not), and internal sites or sites of apparent systemic invasion (including catheter sites). In this isolate breakdown, differences among AFLP types were not significant (likelihood ratio, 13.039; $P = 0.221$).

The delineation of possible host differences among the subtypes was rendered problematic by the superimposed geographic differences among the isolates. Subtypes 1 and 6 contained mainly isolates from Ontario (11 of 13 total isolates), but the majority of isolates from adult skin (11 of 17) also came from the Ontario sample. The subtype 4 isolates from internal sites as well as the internal and neonatal isolates of subtype 5 were from Western Europe (Germany, France, The United Kingdom, and The Netherlands), but that region was the source of only one of the nonneonatal human skin isolates available for study. That isolate belongs to subtype 5. Statistical testing of AFLP type distributions between North America and Europe showed that the differences seen were significant ($P < 0.001$; likelihood ratio, 23.689); however, it was not possible to concomitantly test whether the differences observed were truly attributable to geographic factors rather than to the accompanying differences in host body sites. One *M. furfur* isolate, CBS 6000, clustered separately and was designated type 7. It was from human dandruff sampled in India. The only other Asian isolate in the sample was from adult skin sampled in Hong Kong. It belonged to subtype 6.

M. sympodialis formed a coherent AFLP cluster. The majority of strains were from the backs or chests of healthy subjects from Ontario. Some of the *M. sympodialis* strains studied were from patients who had skin lesions. The lesional and nonlesional isolates showed no genetic differences based on their AFLP types.

Tight clustering was seen among *M. sympodialis* and *M. dermatis* isolates; but in contrast, the clusters of *M. pachydermatis*, *M. globosa*, *M. restricta*, and *M. slooffiae* were relatively loosely structured. The low internal similarity values (Table 1) seen within each of these four species support the impression that they are genetically diverse species.

(ii) Putative new species. There were some strains that fell into a cluster that was not associated with any recognized *Malassezia* taxon (Fig. 1). These strains appear to belong to a species that is as yet undescribed.

LSU rDNA D1 and D2 domain and ITS sequence analysis. Sequence analyses of the LSU and ITS regions resulted in eight well-separated, distinct groups representing the eight different *Malassezia* spp. The D1 and D2 domain and ITS sequences were in the range of 550 and 600 bp, respectively. The nucleotide sequences determined in this study have been deposited in the CBS and GenBank databases, which may be further consulted for identification purposes (www.cbs.knaw.nl/databases/index.htm and www.ncbi.nlm.nih.gov). The phylogenetic trees based on the ITS and LSU sequences were in complete agreement with each other. Some species turned out to be genetically heterogenous with respect to the D1 and D2 domain and ITS sequences. This was true in particular for *M. furfur* (D1, D2, and ITS), *M. sympodialis* (ITS), *M. pachydermatis* (D1, D2, and ITS), *M. globosa* (D1, D2, and ITS), and *M. slooffiae* (D1, D2, and ITS). Phylogenetically, *M. furfur* and *M. obtusa* appear to be sister species. A sister species relationship is also found with *M. restricta* and *M. globosa*. In both trees, the phylogenetic origin of *M. slooffiae* was unclear.

Comparison of physiological and molecular methods. The results of the two molecular methods used in our study were concordant. However, some discrepancies were observed when the AFLP analysis and sequencing results were compared with the results of physiologically based identification. One isolate (98F) that had previously been identified as *M. furfur* was reidentified as *M. sympodialis*, while three isolates (VMB3, GMB1, and WF39) that had previously been identified as *M. globosa* were reidentified as *M. sympodialis*. One isolate (JSB2) that was formerly identified as *M. slooffiae* was reidentified as *M. sympodialis*, and one (SF2) that was formerly identified as *M. sympodialis* was recognized as *M. slooffiae*. Two isolates (GM420 and 4SJCMC1) which had previously been identified as *M. pachydermatis* and *M. globosa*, respectively, were reidentified as *M. furfur*. Overall, a misidentification rate of 13.8% was observed.

DISCUSSION

Increased interest in gaining a better understanding of the epidemiology of *Malassezia* infections has led to the development of epidemiological applications for several molecular typing methods that are able to differentiate *Malassezia* isolates (1, 3, 4, 9, 15–17, 20, 28, 36, 38, 40). The recent identification of two new *Malassezia* species, *M. dermatis* (39) and *M. equi* (32), has further substantiated the need for molecularly specific techniques to distinguish *Malassezia* species. Furthermore, linking the epidemiological and genotyping data will improve our understanding of the *Malassezia* species.

Theelen et al. (40) established AFLP analysis as a useful discriminatory technique for *Malassezia* species identification. AFLP analysis has the capacity to assay a much greater number of loci for polymorphisms than are surveyed by other PCR-based techniques (2) because it is based on the ligation of known sequences (adaptors) to a wide range of restriction fragments; these adaptors then function as targets for PCR primers (2, 34). The use of an internal size standard with every sample for normalization purposes greatly enhances the reproducibility of the results (5). Unlike results from most other PCR-based methods, which analyze only a part of the genome, the banding patterns in AFLP analysis illustrate a broad-ranging subsample of the whole genome and are much easier to analyze than those resulting from restriction fragment length polymorphism and random amplified polymorphic DNA analysis. Another advantage of AFLP analysis is that the patterns can be stored in an accessible general database for future comparison and species identification of additional *Malassezia* isolates.

Sequencing of the two most varied domains, D1 and D2, of the 26S rDNA has proved useful at both the phylogenetic and taxonomic levels, as the region is sufficiently varied to allow distinction between species, in particular basidiomycetes (27, 35). On the other hand, sequencing of the ITS region has also been found to be useful in discriminating closely related species (10, 23). In the case of *Malassezia* spp., Gupta et al. (20) observed that PCR-restriction fragment length polymorphism analysis of the ITS region was sufficient to resolve the differences between the physiologically similar species *M. furfur*, *M. sympodialis*, and *M. slooffiae*. Further, sequence diversity within various species has been observed, which suggests the presence of several genotypes within the species (11, 37). In keeping with these observations, we decided to sequence both of these regions and also to add AFLP analysis to further refine the precision of genotypic clustering in this group and to facilitate accurate species identification.

Following up on the studies of Theelen et al. (40), and testing numerous new clinical isolates, we confirmed that the AFLP analysis yields high-quality fingerprints and is species specific in typing *Malassezia* isolates, though some species contain multiple subtypes. We were able to characterize the major genotypes, including several novel ones, that were present in the *Malassezia* isolates from Ontario. AFLP clusters seen in *M. furfur* in our study suggested the existence of some previously undisclosed specificities. For example, our *M. furfur* genotype 1 isolates came mainly from sites on the chest and back, a remarkable finding given that many of the other *Malassezia* isolates screened came from body sites, such as the exposed areas of the face, that are only subtly physiologically differentiated from the skin of the trunk. In addition, subtypes 1 and 6 appeared to be especially associated with nonneonatal skin, a habitat in which populations of *M. furfur* isolates might be expected, to some extent, to have attained an ecological balance among themselves and with other species (i.e., to be living in a way approximating the ecological k-strategy or "climax community") (7). On the other hand, subtypes 4 and 5 were obtained mostly from sites of internal invasion in immunocompromised patients or from neonatal skin, i.e., from classic newly available sites potentially allowing aggressive, ruderal-type (ecological r-strategy) invasion. Human skin may support such strongly ruderal genotypes in relatively small numbers except in sites of skin disturbance, where they may have an advantage. (*M. furfur*, as a whole, with its relatively rapid growth and minimally fastidious character in vitro, may already constitute one of the more ruderal components of the spectrum of skin *Malassezia* species, as would be predicted from its regular involvement in nosocomial infection.)

We could not rule out the possibility that the distributional differences between the subtype 1 and 6 group and the subtype 4 and 5 group reflected not ecology but rather a difference in geographic distribution or a sampling artifact simulating such a difference. Subtypes 1 and 6 were mainly from the Ontario sample, consisting mainly of isolates from adult skin, and subtypes 4 and 5 were mainly from Western Europe, where available *M. furfur* isolates came mostly from in-hospital studies. It appears highly unlikely that Ontario could have strongly geographically specific *Malassezia* populations in contradistinction to those of Western Europe, since most Ontarians are of western European origin within their own lifetime or within a small number of generations. Without extensive follow-up sampling, however, the underlying causality of the distributional differences established among subtypes 1, 4, 5, and 6 cannot be discerned. In particular, a sample of hospital-associated isolates from Ontario and skin isolates from adult western Europeans would be of value. A relatively large sample of *Malassezia* isolates of all species, however, would have to be processed in order to capture sufficient *M. furfur* isolates to resolve the questions raised by the present study. Development of selective isolation media for individual *Malassezia* species would be of value.

The disclosure of the intriguing differences among human-associated subtypes, as well as the finding of two genotypes strongly associated with animal sources, dramatically illustrates the power of AFLP analysis in allowing us to discover potentially important epidemiological patterns within *Malassezia* species. AFLP analysis was also able to discriminate *Malassezia* strains that could not be identified by physiological tests (Fig. 1). As mentioned above, it is probable that these strains belong to an as-yet-undescribed species. Further investigation is in progress.

The discrepancies observed in our study between phenotypic and molecular methods of identification were comparable to those observed by Makimura et al. (28), who used ITS1 sequencing as a molecular identification standard. They observed that out of 46 clinical isolates that had formerly been identified as *M. furfur* by employing the conventional phenotypic approach, 22 were *M. sympodialis* and 5 were *M. slooffiae*. It is worth mentioning, however, that misidentification may not be

the only potential source of such findings. *Malassezia*, as a lipophile, is relatively hydrophobic, and reliable single-cell cultures may be relatively hard to make. Clumping occurs in water, especially if oil from the medium is present, unless relatively vigorous efforts are made. It appears that cultures of slow-growing *Malassezia* spp. may conceal relatively inactive inocula of certain faster-growing species and that when the cultures senesce, the contaminating inocula are activated in a way that causes the slow-growing species to be overgrown. Nakabayashi et al. (31) observed that *M. globosa*, which had been detected in a primary culture, disappeared in several experiments and that only *M. sympodialis* remained in the culture. More than one *Malassezia* species may be present in a clinical sample or even in an apparent single colony on a contact plate, necessitating considerable effort to ensure that each *Malassezia* colony submitted for identification is in a pure state. In some situations, pharmacological selection pressure derived from topical drugs or hygienic materials may also influence the isolation of *Malassezia* spp. from clinical samples. Trace quantities of inhibitors carried over into primary cultures may result in the growth of some species being partially repressed until later subcultures attenuate the inhibitor levels. More research is required to find out whether the discrepancies in identification are due to technological error in the physiological methods or to sampling artifacts as mentioned above.

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