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species have been identified: *M. dermatis* (39), which was isolated from atopic dermatitis patients, and *M. equi* (unpublished), a species that was isolated from the skin of horses (32).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 

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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 Nutton 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, 60%, 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 at 18,300 × g for 20 min at 4°C, and 700 μl of the supernatant was transferred to a fresh microcentrifuge tube. Subsequently, 700 μl of chloroform-isooamyl 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 (Biolsabs, Westburg, The Netherlands). The sequences of the primers EcoRI and MseI were 5′-GA CGTGGCTAAAATTCGCA 3′ and 5′-GATTGAGCTCTGAGTAAC3′, respectively. The adaptors used were EcoRI (5′-CTCGTGAAGTCTCCGTACC-3′, forward; 5′-CTCAGTGCGAGGCTCCCTAAT-5′, reverse) and MseI (5′-GAGCTATC AGCTTCGAGAAGTACC-3′, forward; 5′-GAGCTATCAGCTTCGAGAAGTACC-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 MgCl₂, 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 19 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 56°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 Biomimetics 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.

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 clusters. 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

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<th>Origin</th>
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<th>AFLP subtype</th>
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<th>LSU accession no.</th>
<th>% Similarity of ITS sequences</th>
<th>% Similarity of LSU sequences</th>
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| M. globosa | Pityriasis versicolor | United Kingdom | Human | AY387132 | AY387228 | 100 | 100 |
| CBS 7966 T* | Dandruff | United Kingdom | Human | AY387133 | AY387229 | 92.9 | 99.82 |
| CBS 7874 | Skin | Hong Kong | Human | AY387134 | AY387230 | 95.49 | 100 |
| HK10 | Scalp | Canada | Human | AY387135 | AY387231 | 94.98 | 99.82 |
| MPS3 | Chest | Canada | Human | AY387136 | AY387232 | 95.37 | 99.82 |

| M. obtusa | Healthy skin | United Kingdom | Human | AY387137 | AY387233 | 100 | 100 |
| CBS 7876 T, GM 215 | Atopic dermatitis | United Kingdom | Human | AY387138 | AY387234 | 100 | 100 |
| CBS 7968 | Ear of dog with otitis externa | Sweden | Dog | AY387140 | AY387236 | 100 | 100 |
| CBS 1885 | Ear of dog with otitis externa | Sweden | Dog | AY387141 | AY387237 | 95.27 | 99.46 |

| M. pachydermatis | Healthy skin | United Kingdom | Human | AY387143 | AY387239 | 100 | 100 |
| CBS 7877, T* | Healthy scalp | Canada | Human | AY387144 | AY387240 | 99.85 | 100 |

| M. restricta | Healthy skin | United Kingdom | Human | AY387145 | AY387241 | 90.32 | 99.46 |

| M. slooffiae | Healthy ear | France | Pig | AY387139 | AY387235 | 100 | 100 |
| 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*
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 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

<table>
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<th>Isolate</th>
<th>Origin</th>
<th>Location</th>
<th>Host</th>
<th>AFLP subtype</th>
<th>ITS accession no.</th>
<th>LSU accession no.</th>
<th>% Similarity of ITS sequences</th>
<th>% Similarity of LSU sequences</th>
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| M. dermatis | CBS 9145 | Atopic dermatitis | Japan | Human | AB070360 | AB070365 | 100 | 100 |
| CBS 9160 | Atopic dermatitis | Japan | Human | AB070356 | AB070361 | 100 | 100 |
| CBS 9170 | Atopic dermatitis | Japan | Human | AB070358 | AB070363 | 100 | 100 |

Unknown species

| unknown species | ISB2 | Back | Canada | Human | Unknown | Unknown | Unknown | Unknown | Unknown |
| unknown species | LNS2 | Scalp | Canada | Human | Unknown | Unknown | Unknown | Unknown | Unknown |
| unknown species | KHS2 | Scalp | Canada | Human | Unknown | Unknown | Unknown | Unknown | Unknown |
| unknown species | BSB1 | Back | Canada | Human | Unknown | Unknown | Unknown | Unknown | Unknown |
| unknown species | TPF2 | Forehead | Canada | Human | Unknown | Unknown | Unknown | Unknown | Unknown |
| unknown species | DRRC2 | Chest | Canada | Human | Unknown | Unknown | Unknown | Unknown | Unknown |
| unknown species | 4GMC1 | Chest | Canada | Human | Unknown | Unknown | Unknown | Unknown | Unknown |

*a Asterisks indicate the type strains used for determining similarity values.

*b AFLP subtypes are shown for M. furfur only.
FIG. 1. AFLP analysis banding patterns of *Malassezia* species. Unknown species, strains not associated with any recognized *Malassezia* taxon.
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. pachydermatis* (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 4JJC1) 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.

<table>
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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-range 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 neonatal 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.

REFERENCES