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Molecular Analysis of Fungal Microbiota in Samples from Healthy Human Skin and Psoriatic Lesions

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Psoriasis, a common cutaneous disease of unknown etiology, may be triggered by infections, including those due to fungi. Since the fungal community of human skin is poorly characterized, we aimed to analyze the mycological microbiota in healthy skin and psoriatic lesions. Twenty-five skin samples from five healthy subjects (flexor forearm) and three patients with psoriasis were analyzed using broad-range 18S ribosomal DNA (rDNA) and 5.8S rDNA/internal transcribed spacer 2 (ITS2) *Malassezia*-specific PCR primers. Broad-range PCR analysis indicated that most organisms resembled *Malassezia*. *Malassezia*-specific 5.8S/ITS2 analysis of 1,374 clones identified five species and four unknown phylotypes, potentially representing new species. The species distribution appears largely host specific and conserved in different sites of healthy skin. In three subjects, the *Malassezia* microbiota composition appeared relatively stable over time. Samples of *Malassezia* microbiota from healthy skin and psoriatic lesions were similar in one patient but substantially different in two others. These data indicate the predominance of *Malassezia* organisms in healthy human skin, host-specific variation, stability over time, and as yet, no consistent patterns differentiating psoriatic skin from healthy skin.

Human skin is colonized by diverse microbiota, including bacteria and fungi, that can be pathogenic under particular circumstances (14, 16). Traditionally, microorganisms have been identified by culture-dependent methods; however, many species are fastidious and underrepresented in cultures from mixed microbial communities (13), whereas others cannot be cultivated under known conditions (2). Therefore, culture-independent molecular techniques have been used for the identification of microbial species within ecosystems (2, 9, 27, 42).

Such methods, particularly the analysis of rRNA genes, have been employed to characterize bacterial and fungal communities associated with diverse human body sites, including intestine (11), gingiva (28, 33, 43), esophagus (45), vagina (65), and outer ear canal (13). As predicted, these studies revealed greater diversity, including previously undescribed organisms, than did previous analyses based on culture-dependent techniques.

The application of molecular techniques has been advocated to characterize the microbiota in both healthy and diseased skin (14). To date, rRNA data have been used to identify species associated with fungal dermatoses (21, 29, 38, 39) and PCR-based diagnostic tests have been developed (15, 26, 62). Psoriasis, a common dermatosis affecting about 3% of the population in industrialized countries (3), is characterized by erythrosquamous cutaneous lesions associated with abnormal patterns of keratinocyte growth and differentiation (35). Although of unknown etiology, trigger factors, including physical trauma and streptococcal infections, may provoke clinical manifestations (51). Fungal organisms, including *Candida albicans* (63) and *Malassezia furfur* (3), have also been associated with the development of psoriatic skin lesions, and differences have been observed in the *Malassezia* species distributions in healthy subjects and patients with psoriasis (23, 24, 46).

The aims of this study were to use molecular methods to identify the fungal species present in human skin, understand specificity by host and time, and compare the populations present in health and in psoriatic lesions.

MATERIALS AND METHODS

Subjects and sample collection. Five healthy subjects (two males, three females; age 21 to 54; mean, 35.2 ± 11.3 years) and three subjects with psoriasis (three males; age 34 to 55; mean, 47.7 ± 11.8 years) were analyzed. All subjects provided written informed consent approved by the NYU Institutional Review Board. From each healthy subject, at least two samples were obtained from the left and right forearms and, for two subjects, another sample was obtained from each forearm 10 months after the first. From each patient with psoriasis, at least three skin samples, including unaffected skin and two or three samples from psoriatic lesions, were studied. From a patient with psoriasis (designated patient 1P), samples included two from the same digital lesion obtained six months apart and one from an elbow lesion. For the other patients, two or three separate lesions from different body sites were analyzed (for patient 2P, arm, leg, and forearm; for patient 3P, elbow and leg). Lesions differing in the extent of erythema, swelling, and scaling were chosen. No patient had ever received therapy for psoriasis. Samples were obtained in a DNA-free clean room by rubbing the skin using two sterile cotton swabs soaked in ST solution (0.15 M NaCl with 0.1% Tween 20). The head of each swab was aseptically cut from the handle, placed into a microcentrifuge tube containing 100 μl of ST solution, centrifuged for 5 min, and then removed. To detect possible contamination, negative controls were prepared using cotton swabs in ST solution without any contact with skin and then subjected to the above-mentioned procedures.

DNA isolation. Total genomic DNA was extracted by adding an equal volume of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl, 2% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K to the centrifuged ST solution. After an overnight incubation at 55°C, proteinase K was inactivated by boiling for 5 min, glass beads (1.0 mm diameter) were added, samples were vortex mixed for 3 min, and phenol-chloroform DNA extraction was performed (49). The DNA was precipitated by incubation with 2.5 volumes of absolute ethanol at −20°C for >3 h and then centrifuging for 20 min. The DNA pellets were washed once with 70% ethanol, allowed to dry, and resuspended in 15 μl sterile distilled water.

PCR amplification. Universal PCR primers for fungi were used to amplify the 18S rRNA gene (Fig. 1A). Broad-range fungal primers were chosen based on

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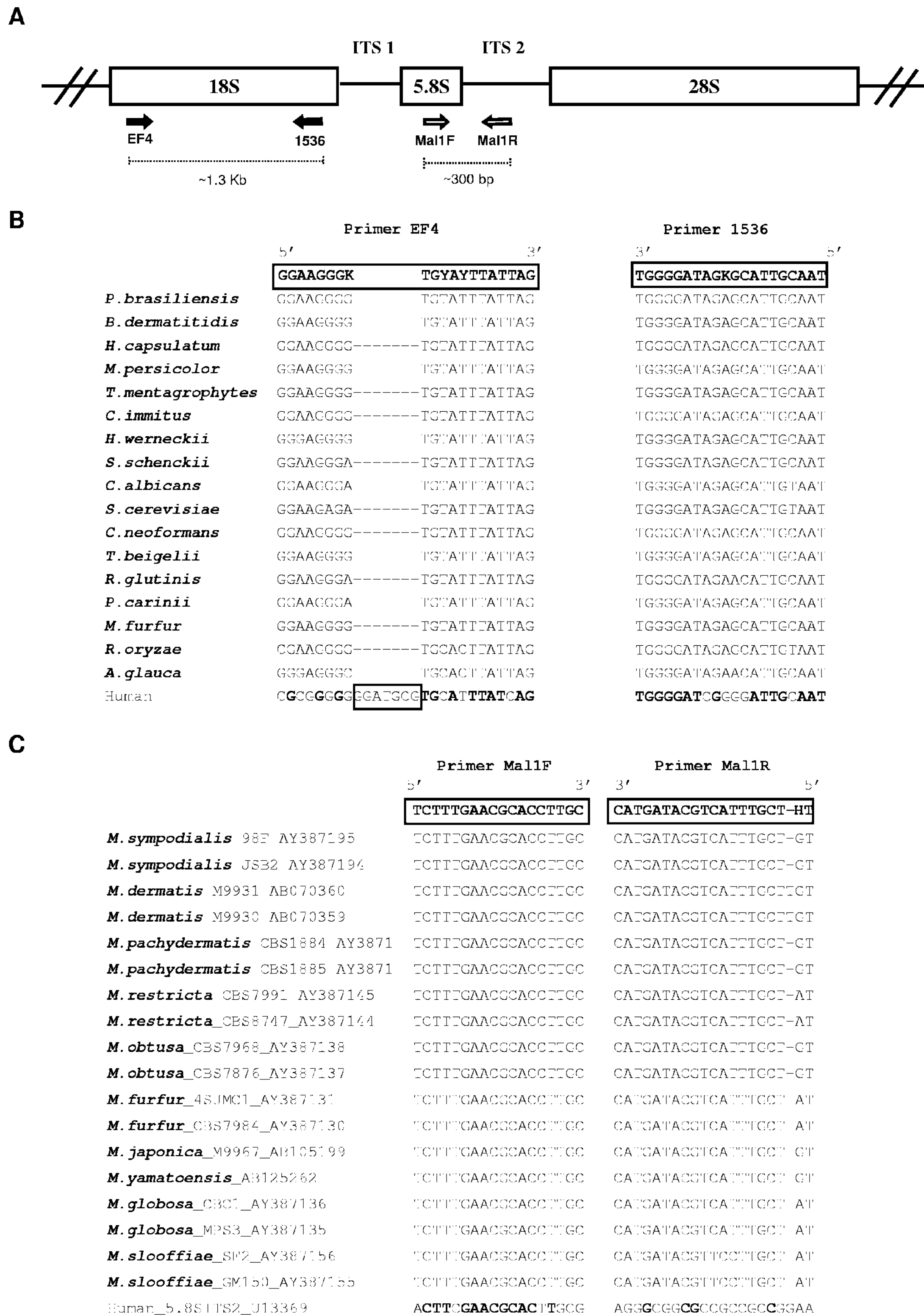

FIG. 1. Fungal loci and sequences related to this study. (A) Schematic representation of the fungal ribosomal gene cluster, with PCR primers indicated (arrows). (B) Pairwise alignment between fragments of the 18S rRNA gene from human and representative fungal species, showing the sequences of primers EF4 and 1536 (long boxes). The human sequence contains a 7-bp insertion within the region represented by primer EF4 (small box). K, A/G; Y, T/C. (C) Pairwise alignment between fragments related to the 5.8S rRNA gene and ITS2 from humans and *Malassezia* species, showing the sequence of primers Mal1F and Mal1R. Bold letters in the human sequences refer to conserved nucleotides with the fungal sequences. H, T/C/A.

comparison between 18S ribosomal DNA (rDNA) sequences from humans and from 17 fungal species representing clinically relevant groups from three different phyla (Fig. 1B) aligned using CLUSTAL W, version 1.81 (61). Primer EF4 anneals to a region containing a 7-nucleotide insertion in the human sequence, reducing the likelihood of amplifying human DNA. To specifically study the genus *Malassezia*, a region containing 5.8S rDNA and internal transcribed spacer 2 (ITS2) was amplified using *Malassezia*-specific PCR primers (Fig. 1A), based on pairwise alignment between the rRNA gene cluster (including ITS1, 5.8S, and ITS2) from humans and 18 *Malassezia* strains, representing 10 species (Fig. 1C). The PCRs were performed using 3.5 mM $MgCl_2$, 0.4 μg/μl bovine serum albumin, 0.25 mM of each deoxynucleoside triphosphate, 20 pmol of each primer, 2.5 U *Taq* DNA polymerase (QIAGEN, Valencia, CA), and 5 μl of extracted DNA in a final 50-μl volume. The PCR conditions were 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 53°C (18S rDNA) or 55°C (5.8S rDNA/ITS2), and 30 s at 72°C, followed by 10 min at 72°C. The PCR products were analyzed by electrophoresis on 1% (wt/vol) Tris-acetate-EDTA–agarose gels containing ethidium bromide and visualized under UV light.

Construction of libraries, screening, and sequencing. Each of the PCR products, whether obtained using universal primers or *Malassezia*-specific primers, were excised from Tris-acetate-EDTA–agarose gels and purified using the QIAquick gel extraction kit (QIAGEN), following the manufacturer's instructions. The purified fragments were cloned into pGEM-T easy vector (Promega, Madison, WI) and used to transform *Escherichia coli* DH5α or XL1-Blue cells. Inserts from transformed cells were PCR amplified using vector-specific primers (SP6 and T7) in a 50-μl mixture containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 5 pmol of each primer, and 1.25 U *Taq* DNA polymerase (QIAGEN). The PCR conditions were 2 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 50°C, and 90 s at 72°C and then 20 min at 72°C, and the products were subject to purification (QIAGEN) according to the manufacturer's instructions. Five-microliter aliquots from the purified PCR products from the 18S rDNA libraries were digested with HaeIII (New England Biolabs, Ipswich, MA) for 1 h at 37°C, the restriction fragments were separated on 1% agarose gels, and restriction fragment length polymorphism (RFLP) types were identified. A second aliquot of representative PCR products from each one of the RFLP types was used to sequence ~700 bp using primer 1536 (Fig. 1B). The analyses were performed on an ABI 3730 automatic sequencer (Applied Biosystems, Foster City, CA). To identify the species *Malassezia restricta* and *Malassezia globosa* from 5.8S rDNA/ITS2 libraries, PCR products were digested with DraI and XbaI, respectively. Nonrestricted fragments were sequenced using primer SP6 to identify other *Malassezia* species.

Phylogenetic analysis. The 18S rDNA and 5.8S rDNA/ITS2 sequences were compared to those in the NCBI GenBank database by using BLASTN algorithm versions 2.2.8 to 2.2.11 (1) (http://www.ncbi.nlm.nih.gov/BLAST/), and sequences were aligned using CLUSTAL_X, version 1.8 (60). Chimeric and human sequences, corresponding to 5.9% of the clones, were not included in the alignments. Phylogenetic analyses were performed using Mega2 software, version 2.1 (34) (http://www.megasoftware.net). Matrices of distance were calculated using the Jukes-Cantor algorithm (31), and the neighbor-joining method (48) was used to generate phylogenetic trees. To identify the closest relatives of the cloned sequences, phylogenetic trees based on 18S rDNA data also included 16 known fungal species, representing clinically relevant groups. For phylogenetic analysis based on 5.8S rDNA and ITS2 to identify *Malassezia*, 10 sequences corresponding to known *Malassezia* species were included. The reliability of the tree topologies was tested by bootstrap resampling with 500 replicates. Novel phylotypes were defined as sequences with <97% similarity to known species in the public databases.

Estimation of species richness. The species richness of normal and psoriatic skin samples was evaluated with a nonparametric richness estimator, Chao 1 (6), using EstimateS, version 7 (http://purl.oclc.org/estimates).

Double-principal coordinate analysis and cluster analysis. Double-principal coordinate analysis (DPCoA) (44) was used to evaluate the sample diversity and analyze the relationship among samples. This method uses the phylotype dissimilarity matrix of samples to calculate the sample diversity. In this analysis, the dissimilarities between different phylotypes are defined as one, and the resulting sample Rao's diversity coefficient is therefore identical to the Gini-Simpson index. To facilitate the visualization of diversity, the first two orthogonal principal axes were obtained based on the sample dissimilarity and plotted to show the distribution of samples in a two-dimensional space. Unsupervised hierarchical clustering techniques were also used to evaluate similarity among samples based on the sample dissimilarity matrix. To assess the robustness of the clustering, bootstrap resampling was done with 1,000 simulations. The diversity information can be decomposed into within- and between-samples diversity values. This allowed the use of a "pseudo F" statistic (the ratio of within-cluster diversity and between-cluster diversity) to examine the possible clustering phenomena, and significance was evaluated by permutation tests (11).

Nucleotide sequence accession numbers. The rDNA sequences of clones representing novel phylotypes were deposited in the GenBank database with accession numbers DQ119888 through DQ119891.

TABLE 1. Fungal species and phylotypes identified in 190 clones from normal skin and psoriatic lesions from patient 1P

Organism identified (% similarity)	% Clones[a]		
	Normal skin (n = 76)[b]	Psoriasis 1 (n = 61)	Psoriasis 2 (n = 53)
Anguillospora rubescens (99)	0	1.6	0
Trichoderma viride (99)	0	1.6	0
Malassezia furfur (98)	1.3	1.6	1.9
Undetermined[c] phylotype A	44.7	8.2	58.5
Undetermined phylotype B	53.9	86.9	39.6

[a] Psoriasis 1 and 2 samples were obtained from finger and elbow lesions, respectively.
[b] Number of clones analyzed.
[c] Similarity to known organisms, <97%.

RESULTS

Broad-range PCR analysis. We first sought to analyze the complete fungal microbiota in the sampled skin by using broad-range primers to amplify fungal 18S rDNA. Taking advantage of a 7-nucleotide insertion present within the human 18S rDNA sequence but absent from the fungal sequences, the modification of primer EF4 (52), together with primer 1536 (5), was used to amplify expected 1.3-kb 18S rDNA PCR products (Fig. 1A and B) and create libraries of cloned fragments from specimens obtained from normal forearm skin and psoriatic lesions. Clones were screened from three separate libraries using RFLP analysis, and partial (~700 bp) sequences were obtained from representative clones of each RFLP type (defined on the basis of the HaeIII restriction pattern). For each RFLP type, at least two clones were sequenced, which indicated high sequence similarity within each type (data not shown). After phylogenetic analyses were performed, sequences with ≤97% similarity with known GenBank sequences were designated phylotypes, defined based on RFLP type and nearest relative (as determined by BLASTN search) and by phylogenetic affiliation. Sequences included in the same phylotype had ≥97% identity to each other. More than 95% of the clones obtained from the libraries from each of the three skin samples were grouped into two unknown phylotypes (designated A and B) (Table 1), phylogenetically related to *Malassezia furfur* (Fig. 2), but with similarity < 97%. Although detected in both normal and psoriatic skin samples, their prevalence differed among the samples (Table 1). From each sample, only one sequence was similar (98%) to *M. furfur* (Table 1). Sequences with similarity to only *Anguillospora rubescens* and *Trichoderma viride* were found in single clones from a single psoriasis lesion (Table 1). *Anguillospora rubescens* is a freshwater hyphomycete (19) not previously reported as either belonging to the commensal human microbiota or responsible for infections. The genus *Trichoderma* comprises saprophytes, although some species, including *T. viride* (30), have been associated with opportunistic infections in immunocompromised patients (64). Both genera belong to the phylum *Asco-*

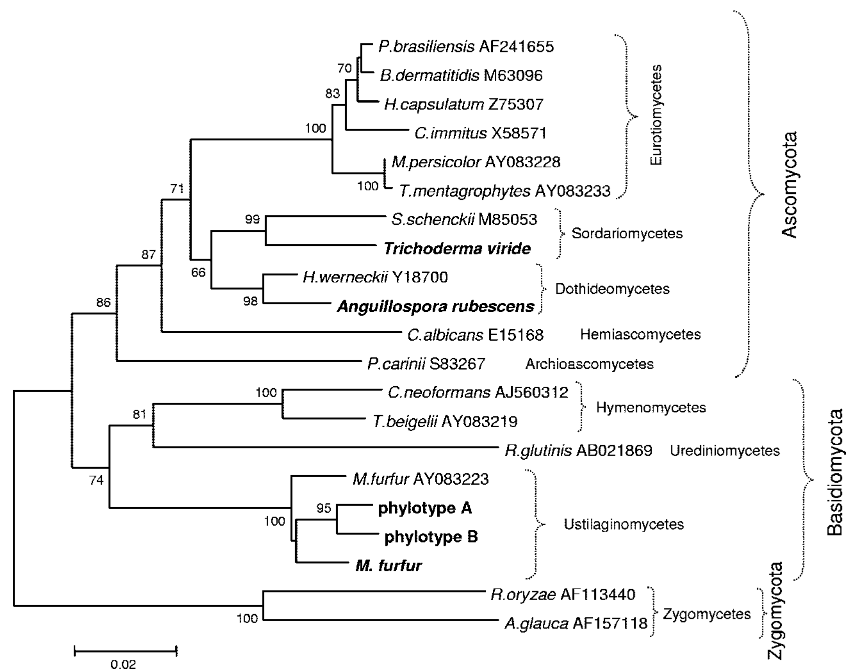

FIG. 2. Neighbor-joining tree based on partial 18S rDNA sequences. The matrix of distances was calculated using the Jukes-Cantor algorithm. Bootstrap values are based on 500 replicates (values of at least 50% are shown). Organisms identified in this study are shown in bold. Codes correspond to GenBank accession numbers.

mycota, whereas the majority of the organisms detected in the samples are in the phylum *Basidiomycota* (Fig. 2).

***Malassezia* microbiota.** Although the most prevalent sequences detected in these skin samples, belonging to phylotype A or B, were phylogenetically related to the genus *Malassezia* (Fig. 2), no species could be assigned because, except for that of *M. furfur*, GenBank does not contain sufficient 18S rDNA *Malassezia* sequences to distinguish among species. Therefore, *Malassezia* sequences in GenBank were evaluated to identify DNA regions that could be used for species-level identification. Sequences corresponding to the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene from *Malassezia* organisms are available and contain regions that differ between species but are conserved within a species. By designing primers to specifically amplify a fragment containing the 5.8S rRNA gene and ITS2 from *Malassezia* species, and not from humans (Fig. 1A and C), we studied the *Malassezia* microbiota in skin samples from five healthy subjects and three patients with psoriasis.

Among the healthy subjects, five different *Malassezia* species were identified, as well as four phylotypes distinct from the currently recognized species (Table 2). *Malassezia restricta* was the most commonly detected species in both samples from two subjects (designated 1N and 3N), whereas *M. globosa* predominated in one subject (2N). Phylotypes 1, 2, and 3, most closely related to *M. restricta*, and phylotype 4, related to *Malassezia sympodialis* (Fig. 3), may in fact represent new *Malassezia* species. Similar to results obtained with the healthy subjects, in the patients with psoriasis, three different species and one unknown phylotype were identified (Table 3); however, the relative clone abundances differed.

Negative controls, prepared using cotton swabs without any skin contact, did not generate detectable signals of amplification by electrophoresis; however, gel fragments were excised at locations where products should migrate, and libraries were constructed and screened. Several insert-containing colonies were isolated, ranging from approximately 3-fold to 100-fold fewer colonies, compared to those of the actual skin samples, with identified species corresponding to some of the common organisms found in skin samples (data not shown). Although probably due to cross-contamination between samples, such contamination has low impact on the results, considering the high ratio between numbers of colonies from skin samples and from negative controls. Contamination by human DNA was a function of the source of the specimen. Only 2 (0.2%) of 826 clones from normal skin samples of healthy hosts had human sequences, and similar results were observed in normal skin samples from patients with psoriasis (0.7% of 152 clones). In contrast, 49 (10.8%) of 454 clones from psoriatic lesion samples contained human DNA ($P < 0.0001$, compared to normal skin samples).

To determine the extent to which the data reflect the diversity of the samples, the species richness of normal and psoriatic skin samples was evaluated using the Chao 1 estimator (6). Based on these analyses, 100% of the estimated species number was detected in both normal and psoriatic skin samples, indicating the strength of the methodology.

Variability of the *Malassezia* microbiota. To evaluate similarities in the distributions of *Malassezia* organisms between skin samples, cluster analyses were performed. The samples were grouped based on the similarities in percentages of the *Malassezia* species, following a procedure used for analysis of gene expression data (12), and the reliability of clustering results was assessed by bootstrap resampling. The skin samples

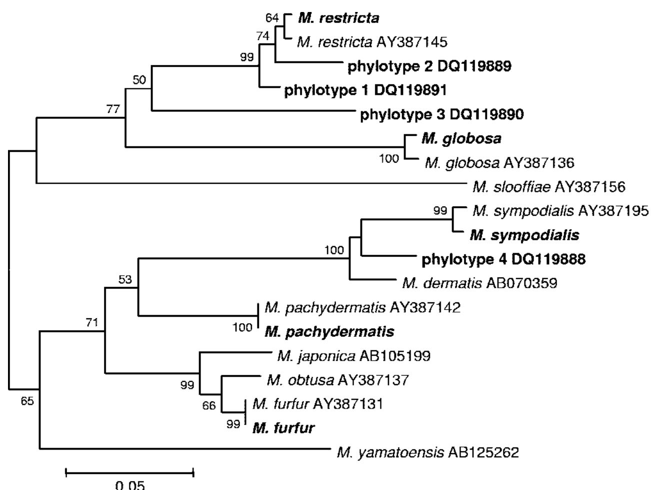

FIG. 3. Neighbor-joining tree based on 5.8S rDNA and ITS2 sequences, showing the relationships among *Malassezia* organisms. The matrix of distances was calculated using the Jukes-Cantor algorithm. Bootstrap values are based on 500 replicates (values of at least 50% are shown). Organisms identified in this study are shown in bold. Codes correspond to GenBank accession numbers.

were also compared using DPCoA. Samples from the same body site in the same host obtained 6 or 10 months apart clustered together in four of five cases (Fig. 4A and B), indicating that the *Malassezia* microbiota are relatively stable over that time period. The proximity between samples from the same body site within an individual obtained 6 or 10 months apart was also verified by DPCoA (Fig. 5) and confirmed by statistical hypothesis testing ($P = 0.05$). In most of the healthy subjects, samples from the left and right forearms were closely related to each other (Fig. 4A and 5), suggesting that the distribution of *Malassezia* organisms is conserved in parallel skin sites and host specific. However, samples from subject 4N grouped in separate clusters, consistent with substantial differences in species distribution (Table 2). Samples from psoriasis patient 1P were closely related to each other, despite originating in healthy or psoriasis-involved skin (Fig. 4B and 5), but greater differences were observed among samples from patients 2P and 3P. Considering both the healthy subjects and patients, hypothesis testing confirmed that samples from an individual subject are closer together than samples from different subjects ($P < 0.0001$). In addition, DPCoA revealed that samples from healthy skin largely overlap with those from psoriatic lesions (Fig. 5) and a separation between these groups could not be confirmed by hypothesis testing ($P = 0.78$).

DISCUSSION

Here we report a broad-range analysis of the fungal microbiota in healthy forearm skin and psoriatic lesions by culture-independent methods. Initially, using universal primers to amplify fungal 18S rDNA from skin samples from a patient with psoriasis, most sequences appeared to represent the genus *Malassezia*. This genus has been described as part of the commensal skin microbiota, but it also has been associated with pityriasis versicolor, seborrheic dermatitis, atopic dermatitis,

TABLE 2. *Malassezia* species and phylotypes identified in 824 clones from normal skin samples from five subjects[a]

Organism identified (% similarity)	% Clones														
	1N				2N				3N		4N		5N		Total (n = 824)
	L-Jan (n = 72)[b]	L-Nov (n = 49)	R-Jan (n = 70)	R-Nov (n = 50)	L-Jan (n = 46)	L-Nov (n = 53)	R-Jan (n = 49)	R-Nov (n = 52)	L (n = 74)	R (n = 73)	L (n = 52)	R (n = 69)	L (n = 65)	R (n = 50)	
Malassezia restricta (99–100)	77.8	87.7	42.9	82.0	4.3	32.1	18.4	40.4	51.4	45.2	0	33.3	13.8	22.0	40.4
Malassezia globosa (98–100)	13.9	4.1	21.4	14.0	89.1	62.3	46.9	57.7	14.9	2.7	0	7.2	6.2	22.0	23.5
Malassezia sympodialis (99–100)	1.4	4.1	28.6	2.0	0	3.8	16.3	1.9	4.1	8.2	96.2	7.2	15.4	30.0	15.0
Malassezia pachydermatis (97–99)	0	0	0	0	0	0	0	0	0	2.7	0	1.4	40.0	0	3.5
Malassezia furfur (100)	0	2.0	0	0	0	0	0	0	0	0	0	0	0	0	0.1
Undetermined[c] phylotype 1	0	2.0	1.4	2.0	6.5	1.9	8.2	0	28.4	41.1	3.8	50.7	24.6	26.0	15.5
Undetermined phylotype 2	6.9	0	4.3	0	0	0	0	0	0	0	0	0	0	0	1.0
Undetermined phylotype 3	0	0	1.4	0	0	0	0	0	1.3	0	0	0	0	0	0.2
Undetermined phylotype 4	0	0	0	0	0	0	10.2	0	0	0	0	0	0	0	0.6

[a] All samples were obtained from flexor surface of forearm in subjects 1N to 5N. L, left arm; R, right arm. Samples L-Jan/R-Jan and L-Nov/R-Nov were obtained in January 2004 and November 2004, respectively.
[b] Number of clones analyzed.
[c] Similarity to known organisms was <97%.

TABLE 3. *Malassezia* species and phylotypes identified in 550 clones from healthy skin and psoriatic lesions from three patients with psoriasis[a]

Organism identified (% similarity)	% Clones										
	1P				2P				3P		
	Normal skin (n = 72)[b]	Psoriasis 1A (n = 76)	Psoriasis 1B (n = 50)	Psoriasis 2 (n = 67)	Normal skin (n = 53)	Psoriasis 1 (n = 38)	Psoriasis 2 (n = 43)	Psoriasis 3 (n = 48)	Normal skin (n = 26)	Psoriasis 1 (n = 35)	Psoriasis 2 (n = 42)
Malassezia restricta (98–100)	30.5	50.0	56.0	38.8	0	15.8	13.9	0	0	25.7	9.5
Malassezia globosa (98–100)	38.9	15.8	6.0	31.3	39.6	23.7	9.3	0	100	54.3	14.3
Malassezia sympodialis (99–100)	12.5	2.6	0	26.9	0	36.8	20.9	2.1	0	2.9	47.6
Undetermined[c] phylotype 1	18.0	31.6	38.0	3.0	60.4	23.7	55.8	97.9	0	17.1	28.6

[a] Sample psoriasis 1A was obtained in January 2004, and the other three samples from patient 1P were obtained in July 2004. Psoriasis samples from patient 1P were obtained from finger (psoriasis 1A and 1B) and elbow (psoriasis 2) lesions, those from patient 2P were obtained from arm, leg, and forearm (psoriasis 1, 2, and 3, respectively), and samples from patient 3P were obtained from elbow and leg (psoriasis 1 and 2, respectively).
[b] Number of clones analyzed.
[c] Similarity to known organisms was <97%.

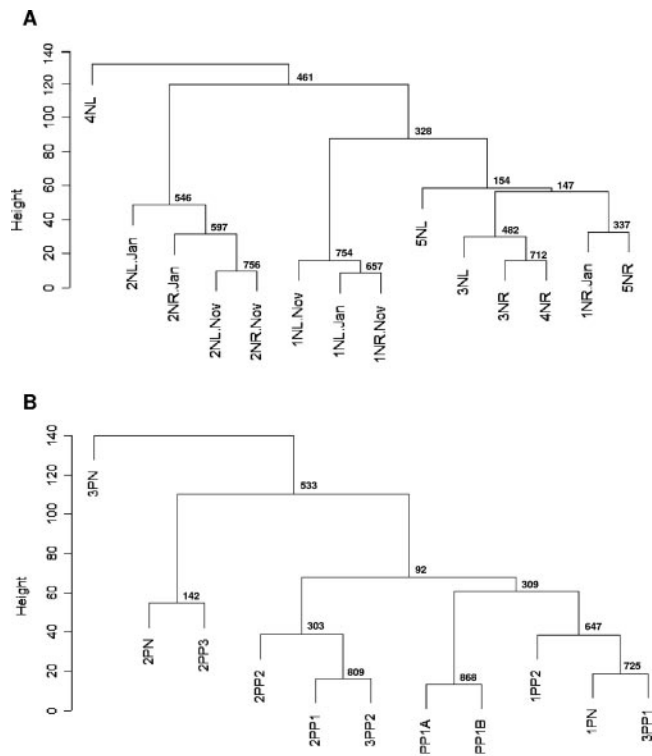

FIG. 4. Consensus tree of hierarchical clustering of skin samples from healthy subjects (A) and patients with psoriasis (B). Height corresponds to Euclidian distance between samples. The number at each node represents the bootstrap value, based on 1,000 iterations. (A) 1N through 5N, healthy subjects; L, left arm; R, right arm. Samples L-Jan/R-Jan and L-Nov/R-Nov were obtained from the same site, 10 months apart. (B) 1P through 3P, patients with psoriasis; N, samples from normal skin; P1 through P3, samples from psoriatic lesions. Samples 1PP1A and 1PP1B were obtained from the same lesion, 6 months apart.

folliculitis, and psoriasis (3, 8, 16, 20). Patients with psoriasis, but not healthy subjects, have been reported to have serum antibodies to *Malassezia* proteins (36, 53) and *M. furfur*-induced, Th1-related cytokines in peripheral blood mononuclear cells (32). *Malassezia furfur* can invade cultured human keratinocytes, modulate proinflammatory and immunomodulatory cytokine synthesis, and affect the expression of cutaneous proteins (4), especially those related to cell migration and proliferation, potentially enhancing inflammation (3).

Because it was not possible to identify *Malassezia* at the species level in our broad-range analysis due to the limited 18S rDNA *Malassezia* sequences available, *Malassezia* species were analyzed using primers to amplify a fragment containing 5.8S rDNA and ITS2. Although the fragment analyzed in this study containing 5.8S rDNA and ITS2 is only ~300 bp, the level of polymorphism between species is sufficient to allow their identification. Using restriction analysis to identify two common species decreases the number of clones to be sequenced, facilitating throughput and lowering costs associated with analysis of the biota. The skin swab technique is simple, allowing large areas to be sampled, and can be used repetitively in serial studies. The contamination by human DNA was more substantial in samples from psoriatic lesions, reflecting the enhanced

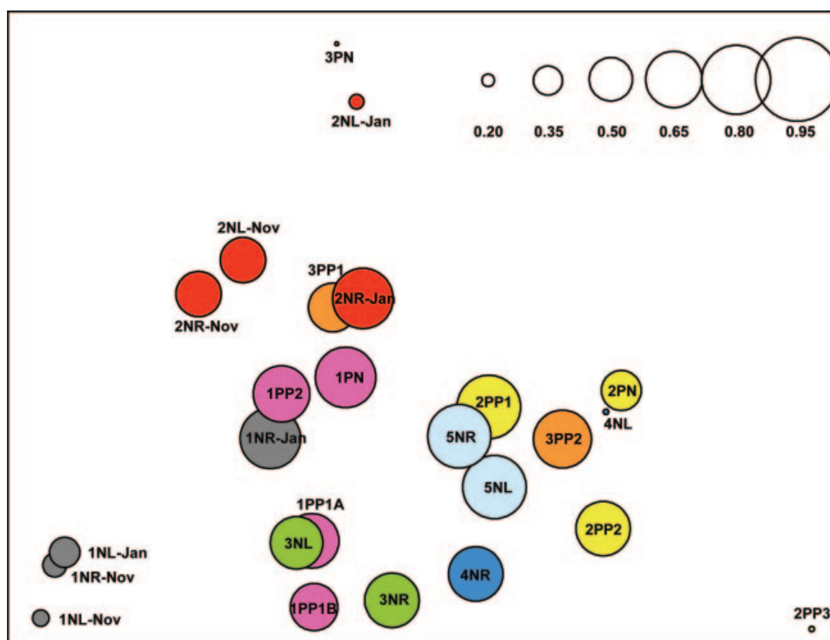

FIG. 5. Scatterplot of the first two orthogonal principal axes based on the sample dissimilarity matrix. The samples from each subject are represented with the same color, and the sizes of the circles are proportional to the sample diversity, as determined by Rao's analysis. The diversity scale is shown in the upper right corner.

cellular proliferation due to inflammation (35), but it represented a small fraction (10%) of the clones. Studies of species richness provide evidence that our sampling of *Malassezia* species in normal and psoriatic skin is at or very near completion. In total, the techniques used may have wider application in studies to determine the distribution of *Malassezia* species in human skin.

In the 25 skin samples from the eight subjects examined, five *Malassezia* species and four unidentified phylotypes were found. Based on the total number of clones, *M. restricta* and *M. globosa* were the most prevalent organisms identified. Both species have previously been detected by molecular (55, 56) and culture-dependent (23, 24, 46, 47) approaches in skin samples from healthy subjects and in patients with dermatoses. *Malassezia sympodialis*, also identified in the samples we studied, has been observed in both healthy subjects and patients with dermatoses (8, 23, 24, 41, 50, 56). *Malassezia pachydermatis*, associated with infections in dogs and other carnivores (10, 18), has been detected in human newborns (7) and in healthy dog owners (40). Although not often isolated from humans, our detection in samples from three (of five) healthy subjects suggests that *M. pachydermatis* may be substantially more common than was previously believed.

Using culture-dependent techniques, *M. restricta*, *M. globosa*, and *M. sympodialis* have been recovered in 38 to 55% of healthy persons and those with psoriasis (23, 24, 46), but their presence in all eight subjects we analyzed suggests a higher prevalence. *Malassezia furfur*, described as common in normal skin microbiota (37), has been associated with psoriasis (3). Using universal fungal primers, only 3 (1.5%) of 190 clones had sequences ≥97% similar to *M. furfur* (Table 1) and analysis using *Malassezia*-specific primers showed only 1 clone (0.07%) of the 1,374 analyzed. It was not detected in a sample from the same body site obtained 10 months later, confirming its low representation at the sites we sampled.

The results of hierarchical clustering analyses suggest that, in the majority of the samples, the *Malassezia* species distributions remained relatively stable over time, were similar among samples from the same healthy subject, and tended to be host specific. Among the three patients with psoriasis, the *Malassezia* microbiota were relatively conserved in samples from one patient, whether from healthy or psoriatic skin, whereas substantial differences were detected among samples from the other two patients, possibly reflecting variation in the microbiota at different body sites. Samples obtained from leg (designated 2PP2 and 3PP2) as well as samples from elbow (1PP2 and 3PP1) grouped together (Fig. 4B), which is consistent with this hypothesis and with a prior culture-based study (23). Although the same species were detected in both healthy and diseased skin from patients with psoriasis, the compositions of the communities may still differ. Genotypes of *M. globosa* (54) and *M. restricta* (56) isolated from patients with atopic dermatitis have been found to differ from those from healthy subjects, suggesting the possibility of genotype-specific roles in pathogenesis that could not be detected by our species-level analyses.

In conclusion, we provide evidence that most fungal organisms in the normal healthy human forearm skin and in psoriatic lesions belong to the genus *Malassezia*. The use of culture-independent methods to identify *Malassezia* organisms is important, since they are fastidious (15, 21, 22). Eleven *Malassezia* species are currently accepted (17, 25, 57–59), with four described since 2002. The four novel *Malassezia* phylotypes detected in this study possibly correspond to new species and should be further characterized, ideally with growth as pure cultures. Analysis of the clones and longitudinal studies indicate host specificity of the distribution of *Malassezia* species.

Thus far, the data do not support the hypothesis that *Malassezia* populations differ in psoriasis and healthy skin.

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